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DIPLOMARBEIT

Titel der Diplomarbeit

Bacterial ghosts as carrier of human hCG-ß-LTB immunocontraceptive antigen

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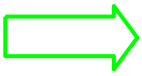
Inhalt

Abbreviations and symbols

µg	microgram
µl	microliter
AB	antibiotics
ATP	adenosintriphosphate
BAP	bacterial alkaline phosphatase
BCCP	biotin carboxyl carrier protein
BG	bacterial ghost
bp	base pair
cfu	colony forming units
CM	cytoplasmic membrane
CPS	cytoplasmic space
CV	immunocontraceptive vaccine
dH_2O	distilled water
DT	diphtheria toxoid
E. coli	Escherichia coli
FSH	follicle-stimulating hormone
fwd	forewards
g	gramm
GnRH	gonadotropin-releasing hormone (=LHRH)
hCG	human choriongonadotropin
hCGß	human choriongonadotropin - subunit ß
hCGß-LTB	human choriongonadotropin – ß fused to heat lab. enterotoxin B
HRP	horst redish peroxidase
hrs	hours
HSD	heterospecies dimer
IM	inner membrane
IPTG	isopropyl-ß-D-thiogalactopyranosid
Kb	kilobasepairs
kDa	kilodaltons
kg	kilogram
l	liter broth
LBv	Luria Bertoni vegetable

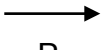
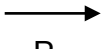
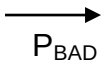
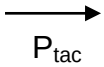
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone (= GnRH)
LPS	lipopolysaccharide
LTB	heat labile enterotoxin B
MBP	maltose binding protein
MCS	multiple cloning site
min	minutes
mg	miligram
ml	mililiter
ng	nanogram
nm	nanometer
OD_{600}	optical density at 600 nm
OM	outer membrane
ON	overnight
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pmol	picomol
PP	periplasm
PPS	periplasmic space
rev	reverse
rpm	rotation per minute
RT	room temperature
SD	Shine-Dalgarno sequence
S-layer	surface layer
sec	seconds
strep	streptavidin
TA	target antigen
TFF	tangential flow filtration
Tm	melting temperatur
TSH	thyroid-stimulating hormone
TT	tetenus toxoid
V	volt
WB	western blot
ZP	zona pellucida

GENES:

	**Gentamycine**	gentamycin resistance gene
	**Kanamycine**	kanamycine resistance gene
	**Ampicilline**	ampicilline resistance gene
	**Mobil. seq.**	gene encoding a mobility protein
	**cI857**	thermosensitive allele of the phage λ-cI-repressor gene
	**Eivb**	C-terminal fusion of the gene encoding the lysis protein E of the bacteriophage ΦX174 and the invivo-biotinylation sequence
	**ara C**	arabinose operon regulatory gene of *E.coli*
	**LacIq**	lacI gene under control of a mutated high expression *lacI* promoter
	**GIII-MCS-myc-His**	GIII-targetsequence - multipe cloning site – Myc – His – construct

	**hCGß-LTB**	human choriongonadotropin – ß fused to heat labile enterotoxin B
	**MBP**	Gene encoding the maltose binding protein (MBP)

PROMOTORS:

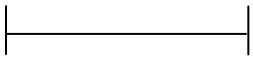
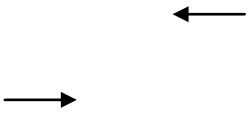
 P_{RM}	**P_{RM}**	repressor maintenance promotor of bacteriophage λ
 P_{mut}	**P_{mut}**	mutated promoter of the PR promoter in bacteriophage λ
 P_{BAD}	**P_{BAD}**	promotor of the *ara*-operon
 P_{tac}	**P_{tac}**	IPTG-inducible tac promotor
 P_{LacIq}	**P_{LacIq}**	a mutated high expression *lacI* promotor

ORIGINS OF REPLICATION:

	**rep**	broad host range origin derived from the *Bordetella bronchiseptica* plasmid pBBR1

	**ori**	origin of replication
	**phage f**	origin of the filamentous bacteriophage f1
	**pBR322**	low copy replication and growth in E. coli
	**M13 ori**	M13 intergenic region, origin of replication
	**pMB1**	origin of high-copy number plasmids derived from pUC-vector series

OTHER SYMBOLS:

	**PCR**	polymerase chain reaction
	**primers**	oligonucleotides for PCR
	**restriction site**	enzymatic DNA restriction at a certain site

1 Zusammenfassung-Summary

1.1 Zusammenfassung

1.1.1 Zielsetzung

In den letzten Jahren beschäftigte sich das Team von Dr. Talwar (Talwar research foundation, New Delhi, India) mit der Herstellung von immunokontrazeptiven Vakzinen (Birth control vaccine) gegen das hCG (human chorion gonadotropin) Hormon, welches eine Schwangerschaft bei sexuell aktiven Frauen verhindern soll. HCG-ß (ß – Untereinheit des hCG) ist ein schwach immunogenes Hormon, welches von Trophoblastenzellen produziert wird. Es ist einerseits für die Implantation des Embryos in die Uterusschleimhaut und andererseits für die Aufrechterhaltung der Schwangerschaft in den ersten Wochen wichtig.
HCG hat als einziger Impfstoff Phase I und Phase II der klinischen Studien an Menschen erreicht und zeigt sehr gute Ergebnisse. Als einziges Problem zeigte sich die geringe Effizienz (60 – 80%). Das bakterielle Ghost (BG)-System bietet sich hierbei als Lösung für dieses Problem an.
BG‘s werden durch kontrollierte Expression des Lysegens E, aus dem Bakteriophagen ΦX174, aus Gram-negativen Bakterien gebildet. Das hierbei exprimierte Protein E führt zu der Bildung eines „Lysis- Tunnel“, der den periplasmatischen Raum verschließt und den Austoss des gesamten Zytoplasmainhalts ermöglicht. Zurück bleibt nur die intakte bakterielle Hülle.
BG’s werden aufgrund ihrer Eigenschaften als Adjuvantien und/oder als Träger fremder, rekombinanter Proteine verwendet.
Das Ziel dieser Arbeit war die Herstellung eines hCG-ß-LTB immunokontrazeptiven Impfstoffs auf Basis des BG-Systems. Die BG’s wurden mittels hCG-ß-LTB rekombinanten *E. coli* K12 produziert. Die hCG-ß-LTB Zielsequenzen wurde vor der Lyse-Induktion exprimiert und in den periplasmatischen Raum transportiert.

1.1.2 Ergebnisse

Die in einen pDrive Klonierungsplasmid eingebauten Zielsequenzen hCG-ß-LTB wurde von Dr. Talwar erhalten (Talwar Research Foundation, New Delhi, Indien).

Durch Klonierung der hCG-ß-LTB Zielsequenz in die MCS (multiple cloning site) des pBGKA Vektors, wird ein neues Expressionsplasmid, pBGK-CGL, hergestellt.
Das pBGKA Plasmid beinhaltet ein GIII-MCS-myc-6xHIS Genkonstrukt, das unter der Kontrolle eines L-Arabinose induzierbaren pBAD Promotors steht. Das GIII Gen kodiert die GIII-Signalsequenz, welche den Transport des fusionierten hCG-ß-LTB-myc-HIS Proteins in den periplasmatischen Raum ermöglicht. Das Zielprotein wird im periplasmatischen Raum zurückgehalten.
Die positiven pBGK-CGL Klone wurden zuerst mittels Anti-myc Antikörper bezüglich Proteinexpression getestet und danach mit dem Lysis Plasmid pGLysivb co-transformiert. Die optimalen Expressions- und Lysisbedingungen wurden mittels einer „expression-lysis study“ ermittelt.
Die rekombinanten BG’s wurden zuerst aus *E. coli* K12 NM522 gewonnen. Die Vorversuche zeigten zwar eine gute Proteinexpression, aber nach Lysis-induktion kam es zu einem Proteinabbau.
Aus diesem Grund wurde die Produktion von rekombinanten BG’s aus *E. coli* K12 W3110 getestet.
Aus beiden bakteriellen Stämmen (*E. coli* K12 NM522 und *E. coli* K12 W3110) wurde jeweils ein Klon mit dem besten Expression-Lysis-Profil ausgewählt und für die Fermentation in einem 30Liter Fermenter verwendet. In den rekombinanten, lyophilisierten BG’s wurde die Konzentration an hCG-ß-LTB bestimmt. Die lyophilisierten BG’s wurden für weitere Fertilitäts-Versuche an Dr. Talwar (Talwar Research Foundation, New Delhi, India) verschickt und werden in Tierexperimenten an ihre anti-hCG Aktivität nach Immunizierung der Versuchstiere getestet.

1.2 Summary

1.2.1 Objectives

In recent years the team of Dr. Talwar (Talwar Research Foundation, New Delhi, India) was engaged with the production of immunocontraceptive vaccine (Birth control vaccine) against hCG (human chorionic gonadotropin) hormone with the aim to prevent pregnancy in sexually active women.
hCG-ß (ß – subunit of hCG) is a weak immunogenic hormone which is produced by trophoblast cells. On the one side hCG-ß is important for the implantation of the embryo in the uterine lining and on the other side it is essential for the maintenance of pregnancy in the first few weeks.
hCG is the only vaccine which has reached phase I and phase II of clinical trials in humans and showed very good results. The only one problem was its low efficiency (60 - 80%). The Bacterial Ghost (BG) system provides in this case an appropriate solution to this problem.
BG's are formed by controlled expression of the lysis gene E from bacteriophage ΦX174, of Gram-negative bacteria. The expression of the E protein leads to the formation of a "Lysis tunnel" which closes the periplasmic space and allows the outflow of the entire cytoplasmic content. Only the intact bacterial envelopes remain. BG's are used due to its properties as adjuvants and/or as carriers of foreign recombinant proteins.
The aim of this work was to produce an hCG-ß-LTB immunocontraceptive vaccine based on the BG system. The BG's were produced using hCG-ß-LTB recombinant *E. coli* K12. The hCG-ß-LTB target sequence was expressed and transported into the periplasmic space before the induction of lysis.

1.2.2 Results

The hCG-ß-LTB target sequence incorporated into pDrive cloning plasmid was constructed by Dr. Talwar (Talwar Research Foundation, New Delhi will, India).
By cloning the hCG-ß-LTB target sequence in the MCS (multiple cloning site) of the pBGKA vector, a new expression plasmid, pBGK-CGL, was produced.

The pBGKA plasmid contains a GIII-MCS-myc-6xHIS gene construct which is under control of an L-arabinose inducible pBAD promoter. The GIII gene encodes the GIII - signal sequence which facilitates the transport of the fused hCG-ß-LTB-myc-HIS protein into the periplasmic space. The target protein is retained in the periplasmic space.

The positive pBGK-CGL clones were first tested for protein expression by using anti-myc antibody and then co-transformed with the lysis plasmid pGLysivb. The optimal expression and lysis conditions were determined using an „expression-lysis study“. First, the recombinant BG's were produced from *E. coli* K12 NM522. The preliminary tests pointed to a good protein expression, but after lysis induction, there was protein degradation.

For this reason, the production of recombinant BG's of *E. coli* K12 W3110 was tested. For both bacterial strains (*E. coli* K12 NM522 and *E. coli* K12 W3110), the clone with the best expression - lysis profile was selected and used for fermentation in a 30 litre fermenter. In the recombinant, lyophilized BG's the concentration of hCG-ß-LTB was determined. The lyophilized BG's were sent on Dr. Talwar (Talwar Research Foundation, New Delhi, India) for further fertility testing in experimental animals.

2 Introduction

2.1 Immunocontraception

Today's world is progressing very quickly. Because of the great buildup of knowledge over the past decades especially in scientific fields like medicine and natural sciences a continous increase in worldwide polpulation could be achieved, particularly in the developing countries like Africa, Asia and South America which causes more and more problems. Not only overpopulution but also the increase of the life-span cause economic as well as social problems. In the case of overpopulation issues for an effective, simple and inexpensive birth control are increasingly important.
In recent years there have been used mainly mechanical and chemical birth control methods, such as - condoms, intrauterine devices, steroids in the form of pills, implants or injection depots. None of these methods are ideal, and without side effects. It is therefore an effort to find new methods for fertility control which should be fast, reliably reversible, high effective, free from systemic and metabolic side effects, inexpenssive, not disturb regularity menstruate or cause extra blood loss. Furthermore it should not block the ovulation or normal production of sexual hormones, it should be easily accessible, applicable at any age of reproductive live and be capable of privat use [1, 2, 8].
This new fertility control method is based on immunocontraception.
Immunocontraception is a birth control method that uses the body's immune response to prevent pregnancy. Nowadays it is used mainly to control populations of wild animals (e.g. elefants, white-tailed deer [5], Eastern Grey kangaroo [4], Brushtail possum [6]) and domestic animals e.g. cats [3], dogs [7], because it's more “humane” than killing and therefore more accepted in company and cheaper than castration.
An immunocontraceptive vaccine (CV) for human must be more effective as a CV for animals. Therefore many scientific teams are working on this issue.
Mammalian reproduction is exquisitely regulated by a cascade of hormones. Invasion of any of these hormones by competent antibodies will interupt fertility [1] (see Fig.1). First of all it’s important to find the correct hormonal pathway which shall be inhibited to avoid undesirable side effects.

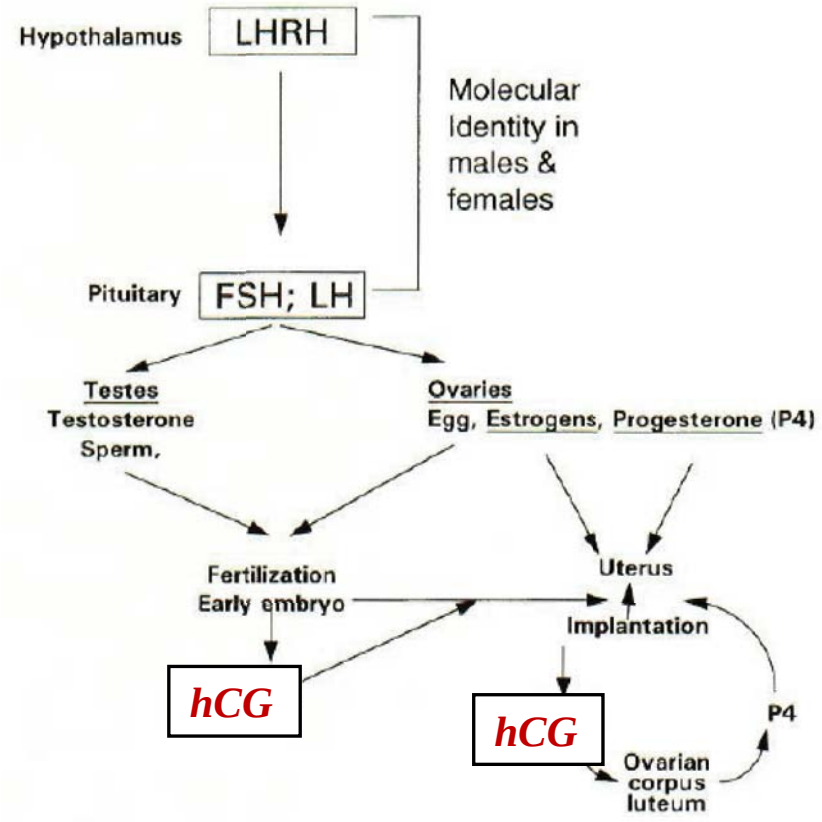


Fig. 1: Cascade of hormones regulating the reproductive system in mammals [1].

2.1.1 Three main Categories of Contraceptive Vaccines [2]

I. immunocontraceptive vaccines targeting gamete production
   - anti-luteinizing hormone / releasing hormone vaccine

II. immunocontraceptive vaccines targeting gamete function
   - antisperm vaccine
   - antioocyte zona pellucida vaccine

III. immunocontraceptive vaccines targeting gamete outcome
   - anti-human chorion gonadotropin (hCG)-vaccine

I. Anti-luteinizing hormone / releasing hormone (LHRH/GnRH) vaccine

This vaccine is intended for both sexes - male and female. The target-homone structure is highly conserved in mammals, therefore the vaccine can be used in different species of animals (domestic pets, farm animals) [9].
Anti-LHRH / GnRH vaccines will be used especially for control of fertility, libido and sex steroid production in companion animals - dogs, and animals raised for meat production - pigs, bulls [2, 9]. This type of vaccine is not suitable for use in humans because it stops the secretion of sex steroids [8, 9] (see Fig. 2).
The anti-LHRH vaccine has also therapeutic applications in sex hormone-dependent cancers – e.g. breast, prostata. According to prostata cancer the anti-LHRH-vaccine reached phase II of clinical trials [1, 2, 9].
A recombinant vaccine based on the BG system carrying the multimeric LHRH or LTB-LHRH in sealed periplasmic space is currently under investigation [10].

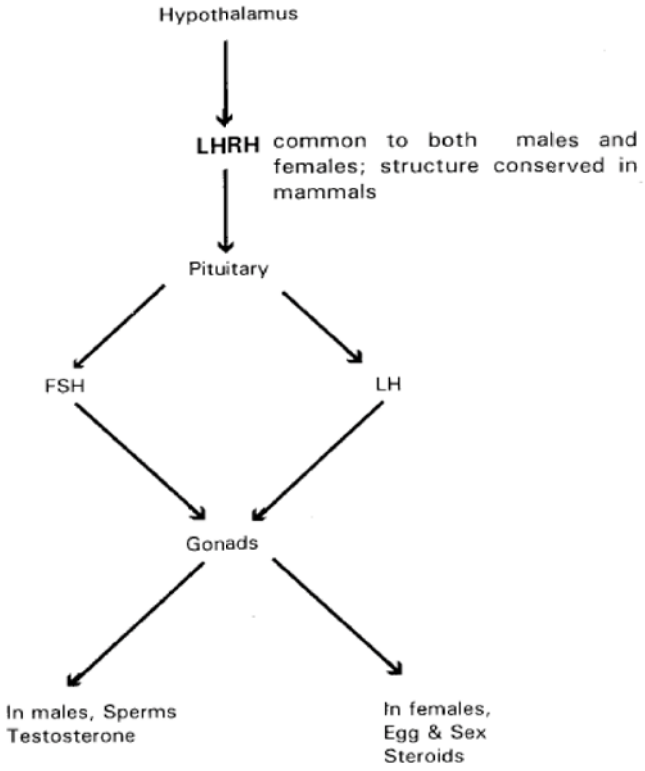


Fig. 2: Luteinizing hormone-releasing hormone (LHRH) *is the key hormone regulating the formation of the gamets and sex steroids via pituitary and gonads. Application: Immunocontraception and immunosterilization in animal species; immunotherapy of prostatic hypertrophy [9].*

II-a. Anti-oocyte zona pellucida vaccine

Zona pellucida (ZP) is an extracellular layer between the egg cell (oocyte) and the follicular epithelium. It consists of glycoproteins, is homogeneous, glassy, serves as a mechanical protection and is equipped with sperm-specific receptors (i.e. only congener sperm can lead to fertilization) [12].
Anti-oocyte zona pellucida vaccines are directed against key proteins of the zona pellucida. Main application potential of ZP-contraceptive vaccines is the control of wild animals and domestic animal populations.
Research is going on for controlling animal populations such as:

- bonnet monkeys [12]
- white-tailed deer [2]
- african elephant [2]
- dogs [2]
- possum in New Zeland [13, 14]

All of these studies show good results but the main problem of the ZP-contraceptive vaccine is to find the right route of administration (delivery of the vaccine), to eliminate possible errors, to make the contraceptive vaccine safer, more effective and cheaper.
A recombinant ZP vaccine based on the BG system, carrying different ZP proteins of brushtail possums, showed promising results. It is under investigation as a bite delivery vaccine in New Zeland [6, 13, 14].

II-b. Anti-sperm - vaccine

At the surface of sperm there are a lot of different structures necessary for the binding of the sperm to the zona pelucida of the oocyte. If the zona pellucida proteins are occupied by antibodies, the sperm can not bind to the oocyte, anymore and therefore fertilization is not possible. Because of that all sperm-ZP binding site proteins are potential anti-sperm vaccine candidates [2].
Anti-sperm vaccines can be applied in both sexes.
Some men are naturaly infertile because they produce antibodies against their own

sperm. In such cases an anti-sperm vaccine has a therapeutic effect. The removal of auto-antibodies leads to an increase in antibody-free sperms, improves the fertility and lead to normal pregnacies and healthy babies [11].

III. Anti – humanchoriongonadotropin - vaccine

Human chorion gonadotropin (hCG) is a special glycoprotein hormone which is not formed constantly and not in all organisms. It is produced by normal trophoblast cells of the placenta during the early pregnancy – and its synthesis starts at the preimplantation stage [1]. It is essential for the implantation of the embryo into the uterus, as marmoset embryos exposed to anti-hCG antibodies failed to implant [1]. hCG can be produced also during trophoblast diseases and by some kinds of tumors. Therefore hCG is also an important tumor marker [1, 2, 15].
hCG shows a heterodimeric structure with an **α-subunit** identical to luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH) and an **β-subunit** unique to hCG consisting of 145 amino acids. The carboxyterminus (~37 amino acids) is unique and weakly immunogenic [1]. Therefore, it is a good candidate for development of a contraceptive vaccine.
To improve the immunogenicity ß-hCG has to be modified either by haptens or by linkage to carriers (tetanus toxoid = TT, diphtheria toxoid = DT, heterospecies dimer = HSD) [1, 9].

2.1.2 Possible applications of hCG-ß

Why is hCGß a good candidate for developing of a contraceptive vaccine [1, 9]?

- hCG is produced under normal conditions only in healthy pregnant women
- hCG can be synthetized by hCG synthetizing tumors
- hCG provides the establishment of pregnancy and its maintenance in the early phase (first trimester) - (see Fig. 3)
- hCG is a diagnostic marker of pregnancy, when hCG synthetizing tumors are excluded
- hCG is inactivated by circulating anti-hCG antibodies

- Primary and secondary structures of hCG-ß are known, so they can be used for bioengineering
- hCG is not involved in the hormone cascade for ovulation and not involved in the production of sex steroids which is a benefit (see Fig.1)

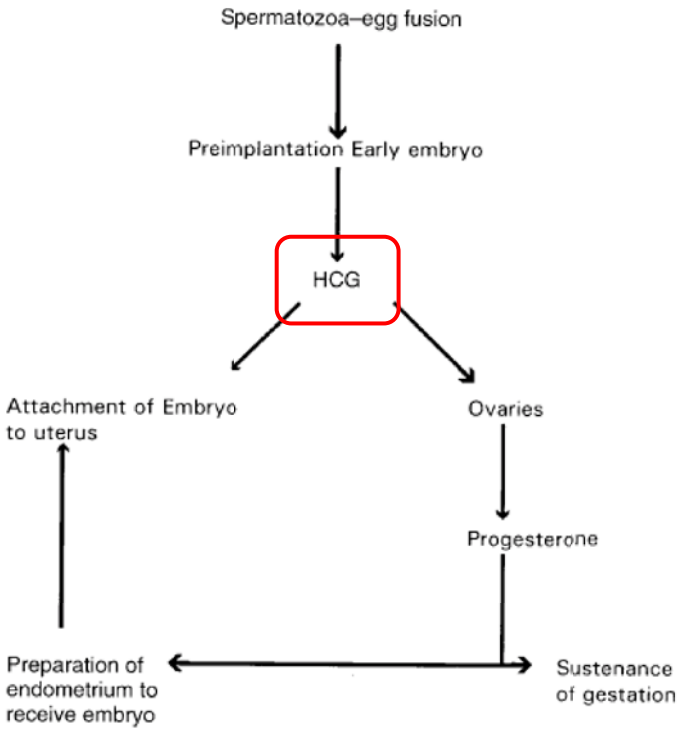


Fig. 3: hCG synthesis and secretion during pregnancy. *hCG is important for both establishment and maintenance of pregnancy [9].*

Application of hCGß-antigen for development of a birth control vaccine.

The anti-hCG vaccine is the first and only CV which has successfully completed Phase I and II of clinical studies [2, 17].
Studies showed that the vaccine is effective when the serum antibody titers go up > 50 ng/ml. To allow pregnancy again, the antibody titer should go down to < 30 ng/ml [2, 16].
During human clinical Phase I and II studies more than 200 women were examinated. The CV was free of side-effects, but generated protective antibody titers only in 60-80% of the examinated women. Since birth control vaccines have to be effective in 90-95%

of the recipients in order to be accetable, further improvements are necessary for the usage of anti-hCG contraceptive vaccine [2, 16].
The main task of the future is to enhance the immunogenicity of hCG by adding of new carriers or adjuvants. The Bacterial Ghost system (BG-system) can serve as both, a new carrier system of hCG as well as a potent adjuvant. The aim of this study is to combine the hCGß protein and the Bacterial Ghost system for development of an effective CV.

Application of hCG antibodies to combat tumors

hCG is secreted not only by chorio carcinomas but also by many other tumor cell types [1, 2, 15]:

- cervical carcinoma
- non-germ cell tumors of bladder, ovary, vulva, prostate, renal, pancreas, lung, colon, oral/facial tissue, stomach, breast.

hCG acts on the tumor cells as an autocrine growth factor. It can be inactivated by anti-hCG antibodies followed by inhibition of tumor growth which leads to necrosis of the tumor [1].

Application of anti-hCG-antibodies as emergency contraception

Today emergency contraception is called "day after pill" and consists of synthetic contraceptive steroids in high dose. The pill must be taken within 48-72 hrs after unprotected sex.
Emergency contraception via anti-hCG antibodies is the method of the future. On this way many side effects can be minimized and the time period for intervention can be extended.
The anti-hCG antibodies would be given within a week of the unprotected sex, to interrupt the implantation. The amount of antibodies requiered would be modest (eg. for a woman of 60 kg it would be around 250 µg) [1].

2.2 Bacterial Ghost System

Bacterial Ghosts (BG's) are non living bacterial cells which consist of an intact envelope with all native surface structures but lack the whole interior of the cell (cytoplasm with all structures, DNA, ribosoms). BG's are produced by expression of the cloned gene E from bacteriophage ΦX174 (E-mediated lysis) in Gram-negative bacteria [18, 19, 25] (see Fig. 4).

2.2.1 E – mediated lysis

Protein E, encoded by lysis gene E of bacteriophage ΦX174, is a very hydrophobic membrane protein of 91 amino acids [20] and can be divided into four domains in which the C- terminus is necessary for the oligomerization and the N- terminus has lytic activity [22, 23, 28] (see Fig. 5).
The E-mediated lysis processes can be divided into three phases [22]. In phase 1 Protein E integrates into the inner membrane by facing the C terminus to the cytoplasmic side (Fig. 5a). In phase 2, due to conformational change of protein E its C terminus is transfered across the inner membrane and assembles into multimers at the potential cell division sites [24]. In phase 3, the C-terminal domain of protein E is transfered towards the surface of the outer membrane of the bacterium. This leads to fusion of the inner and outer membranes and produces a lysis tunnel structure which is 40-200nm in size and localized in the potential zones of cell division (Fig. 5b). Thus, the membrane potential collapses and the whole interior of the cell bursts outwards [21, 22, 27] (Fig. 4).

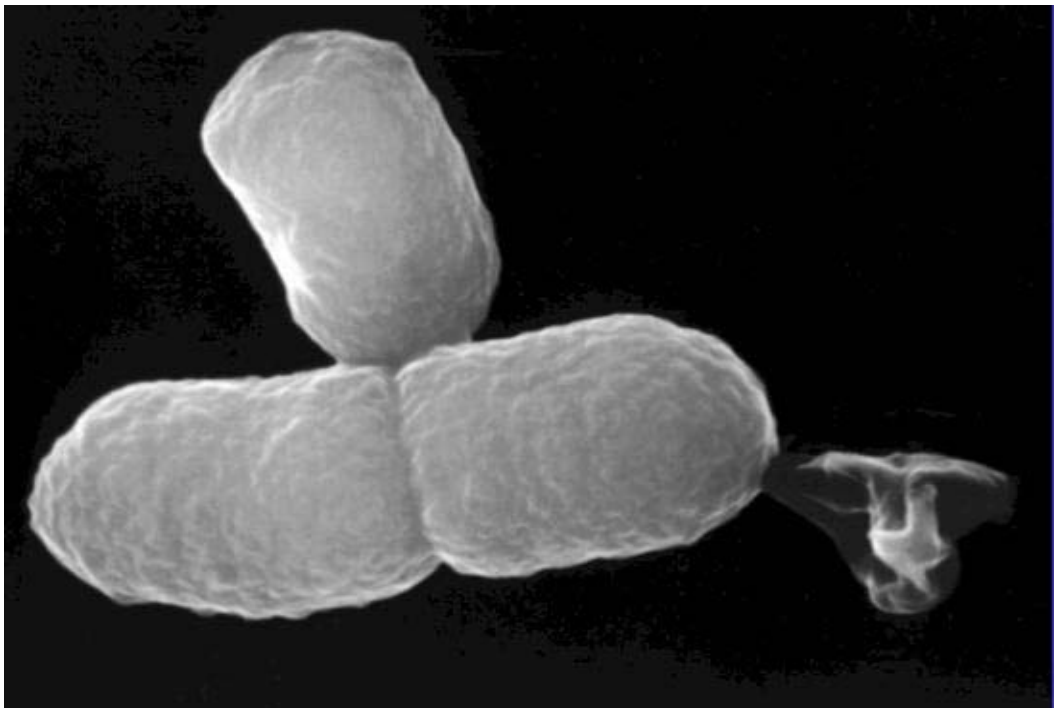


Fig. 4: Bacterial ghost formation of protein E lysed Gram-negative bacteria *[26]. Outburst of the inner cell mass through the E-specific lysis tunnel is showed in scanning electron micrograph.*

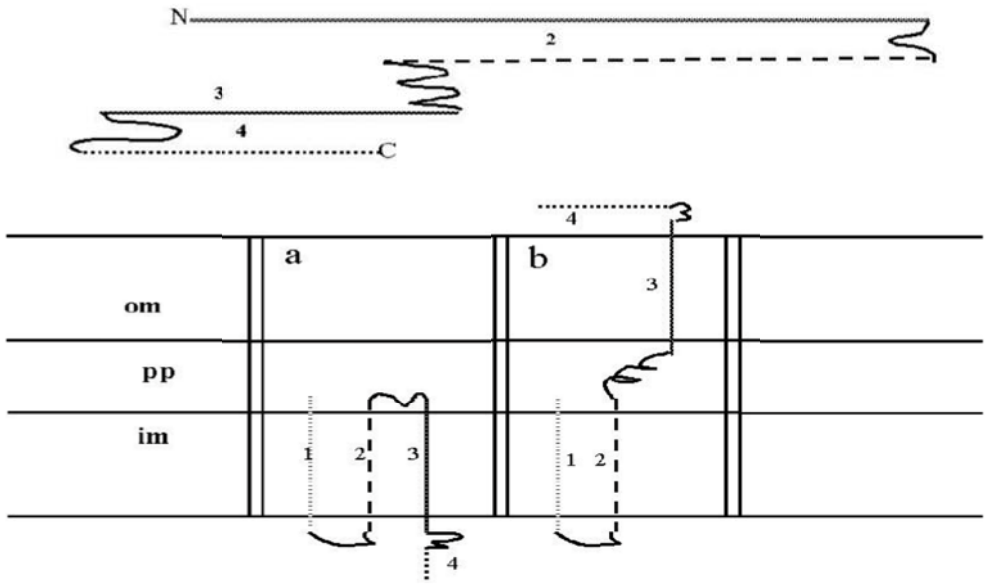


Fig. 5: E-mediated lysis process *[23]*

Phase 1: protein E integrate into inner membrane (a); Phase 2: C-terminus of protein E is transferred across the inner membrane; Phase 3: C-terminus of protein E is transferred towards the outer membrane – production of lysis tunnel (b).

Abbreviations: outer membrane (om), inner membrane (im), periplasm (pp).

2.2.2 Bacterial Ghosts as non-living candidate vaccine

Because of its properties the BG system has a versatile use and can be used as a delivery system for proteins / antigens, nucleic acids, drugs and soluble compounds [35, 36, 37]. Due to its intact surface structures BG can be used as adjuvant and targeting vehicle.
In this work, the BG system is used as carrier and adjuvant for foreign antigen, which is formed prior to lysis and anchored within the BG.

What are the benefits of BG vaccines [35, 38, 39]?

- BG's can be generated from different Gram-negative bacteria
- BG's have native envelopes and no adjuvants are needed
- BG's have intrinsic adjuvant properties
- A simultaneous presentation of multiple antigens (including foreign antigens) is possible
- BG's have a height capacity for foreign antigens
- BG's are very save – no host DNA, no live organisms, no horizontal gentransfer [40]
- The endotoxicity of lipopolysaccharide (LPS) in BG's is not a limiting factor for theirs application as vaccine [41]
- The production is relatively cost-effective in comparison to the more expensive production of purified subunits, synthetic peptides, recombinant bacteria and viruses
- BG's can be stored by room temperature (RT) as freeze-dried material, therefore refrigeration is not needed
- The intrinsic adjuvant properties of bacterial ghosts activate the innate immune system as well as the acquired immune response [47]
- According to the identical surface receptors of BG's with their living counterparts BG's can be used for specific cellular and tissue targeting and thus also for tumor treatment [42]
- The application is very easy – in addition to intramuscular vaccine can be administered oral, nasal, in the form of eye drops or rectal suppository [43].

BG posse a high capacity as carrier for foreign antigens, and can present several antigens simultanesly. The antigens can be incorporated at the following positions [35, 43, 34] (see Fig. 6):

a. Outer membrane (OM) - the antigen is incorporated as a fusion protein of a known outer membrane structure (OmpA or Pilli). A good example is the hepatitis B antigen, which was expressed as an OmpA-fusion in *E. coli* [44].
b. Periplasmic space (PPS) - PPS of BG shows a closed structure and provides the antigen much more protection. Antigens are also immersed in a sugarrich environment of membrane-derived oligosaccharides which protects antigens during lyophilization [34]. The antigen is transported via different secretory systems into the PPS. To achieve this, the antigen is equipped with a specific PPS signalsequence (MalE-or GIII signal sequence). In this work, for periplasmic transport of hCG-ß-LTB antigen, the GIII secretory system was used.
c. Inner membrane (IM) - the antigen is incorporated as a fusion protein with specific anchor sequences for attachment on the inside of the IM. For example, recombinants BG carrying HIV-1 reverse transcriptase [45]. Also DNA can be bound to the IM via membrane anchored DNA-binding proteins such as LacI repressors.
d. Cytoplasmic space (CPS) - The CPS of BG can be filled either with water-soluble subunit antigens or emulsions such that the target antigen itself or a matrix can be coupled to appropriate anchors on the inside of the IM of BG. For example, BG with streptavidin anchored on the inside of the IM can be filled by biotinylated target antigen (TA) [34, 46].
e. S-layer (surface layer) - Expression of the cloned S-layer genes sbsA and sbsB of *Bacillus thermophilus* in *E. coli* leads to accumulation of sheet-like self-assembling SbsA or SbsB proteins in the cytoplasm. The S-layer self assembly products consist of several hundred thousand monomers per cell and they do not form inclusion bodies. Recombinant S-layer proteins rSbsA or rSbsB act as carriers of foreign antigens. When expression of S-layer sheets in *E. coli* is followed by E-mediated lysis, the S-layer structures are retained within the inner lumen of the cytoplasmic space of BG. Target antigens on rSbsA-, rSbsB- carriers can fill the inner lumen, be transported to the PPS or be anchored by membrane anchors on the outer surface of ghosts [48].

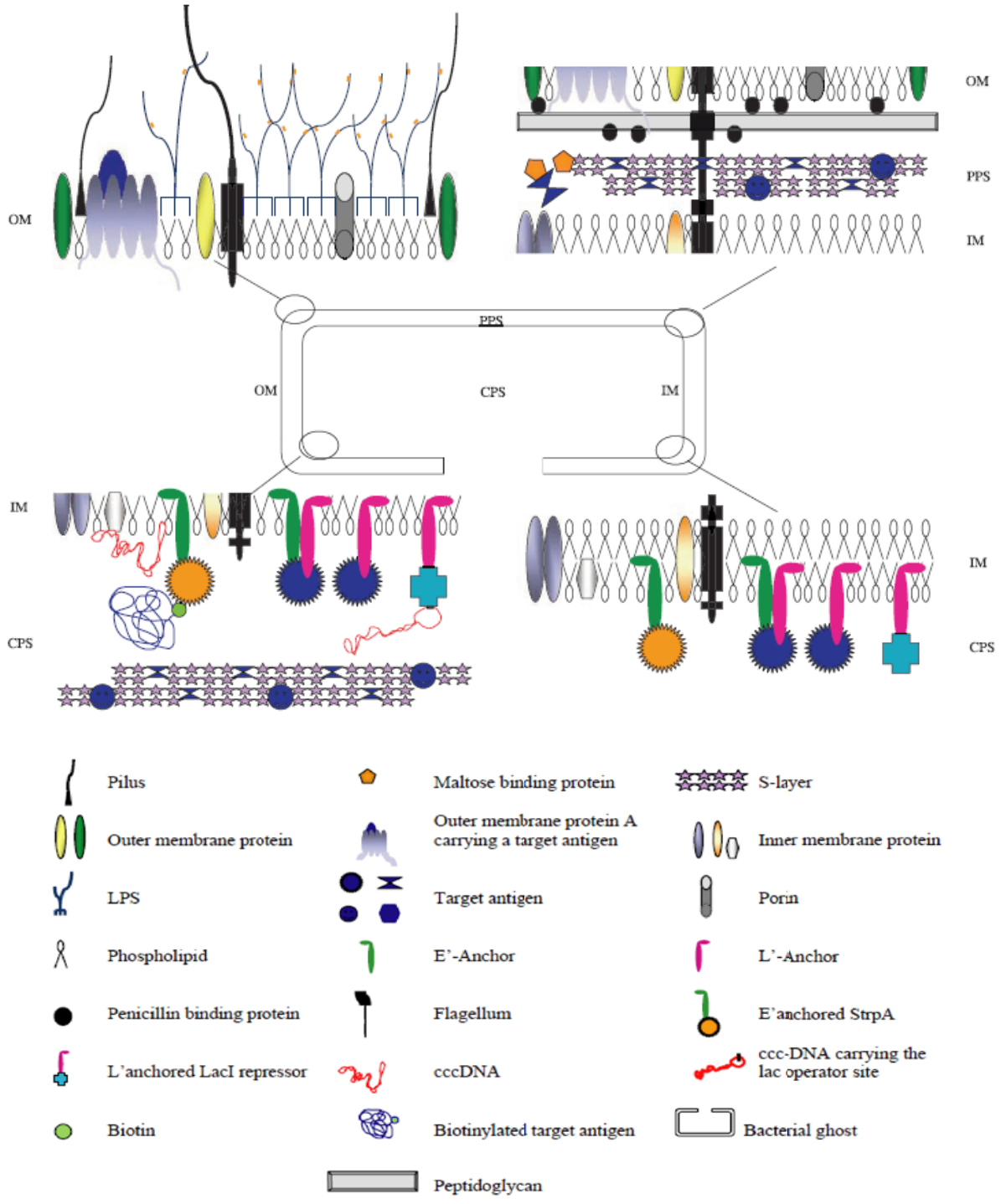


Fig. 6: Schematic diagram of BG as carrier of target antigen *(TA). TA can be localized in four different compartments of BG: 1. OM (outer membrane) as a fusion protein of OmpA; 2. PPS (periplasmic space) as maltosebinding protein or malE-sbsA/sbsB S-layer fusion proteins; 3. IM (inner membrane) anchored via E', L' or E' and L' anchor sequences; 4. CPS (cytoplasmic space) - TA on recombinant SbsA/SbsB- carriers can fill the inner lumen or membrane-anchored StrpA can bind any biotinylated TA to the IM. Adapted from [34].*

2.2.3 **Lysis plasmids**

Monitoring of E-lysis happens by measuring the optical density at 600 nm and by the determination of the live cell counts (i.e. colony forming units = cfu counts). As a result of the E-lysis there is a decrease in the OD_{600} and of the cfu. Protein E expression is also a demonstration for the E-lysis and therefore lysis plasmids carry the Eivb gene. The in vivo biotinylated protein E (Eivb) can be detected using streptavidin coupled to horseradish-peroxidase (Strep-HRP) [50].
Expression of lysis gene E, can be placed under transcriptional control of either the thermosensitive λpL/pR-cI857 or under chemical inducible promoter repressor systems, like lacPO-lacIq or the tol expression system. Several E-specific lysis plasmids with different resistance markers, origins of replication and gene E expression control systems have been published [29, 34].

In different lysis plasmids the gene E expression is controlled by the rightward phage λpR promotor and the corresponding temperature-sensitive repressor, cI857, which is inactivated at temperatures higher than 30°C. Bacterial lysis is induced by a temperature shift of the growing culture from 28°C to 42°C [30, 31]. Mostly it is favorable to cultivate the bacteria at temperatures higher than 28°C. Therefore it was important to extend the heat stability of the λpR-cI857 promotor repressor system. A mutation in the OR2 operator region of the rightward λpR promotor increased the temperature stability of the λpR-cI857 gene expression system. Thus gene E expression is repressed at temperatures up to 36°C and cell lysis is induced at 38°C or higher [31].

pGLysivb lysis plasmid

In this work lysis plasmid pGLysivb was used for production of BG's carrying gene Eivb (in vivo biotinylated protein E) under control of the mutated heat inducible λpR-cI857 promotor repressor system. Additionally pGLysivb contains a gentamycin resistance cassette and a mutated mobilization gene sequence with reduced mobilization activity [32] (see Fig. 7).

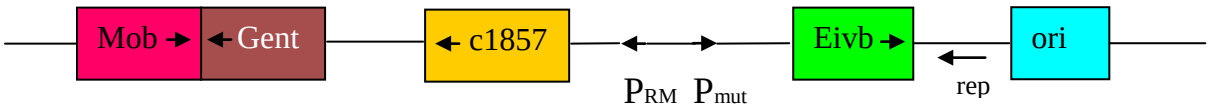


Fig. 7: Map of pGLysivb lysis plasmid *[6201 bp]. Mob: mobilization gene; Gent: gentamycin resistance cassette; Eivb: protein E fused to in vivo biotinylation sequence, which is under control of the mutated heat inducible λpR-cI857 promotor repressor system.*

New lysis plasmids

Amongst others the task of this work was the design and testing of three new lysis plasmids (pGLysivb-2x, pGLMivb, pGULMivb).

I. pGLysivb-2x lysis plasmid

pGLysivb-2x is a pGLysivb derived lysis vector with a second E-specific lysis cassette which is under control of the mutated λpR-cI857 promotor repressor system (see chapter 4.2.1.).

II. pGLMivb lysis plasmid

pGLMivb is a pGLysivb derived lysis vector with an E-specific lysis cassette which is controlled by chemical inducible LacIq repressor / Ptac promoter system (lysis-induction with IPTG) (see chapter 4.2.2.).

III. pGULMivb lysis plasmid

pGULMivb is a pGLysivb derived lysis vector with E-specific lysis cassette which is controlled by the chemical inducible LacIq repressor / Ptac promoter system (lysis-induction with IPTG).
This lysis plasmid was designed for *Neisseia species* and therefore it includes a 10bp-Uptake-sequence (5'- GCCGTCTGAA- 3') (see chapter 4.2.3.).
Neisseria species are known to be transformed less readily if transforming DNA competes with DNA containing the 10-bp sequence GCCGTCTGAA. It has been

postulated that the 10-bp sequence is a recognition sequence which is required for efficient DNA uptake [33].

2.2.4 Expression plasmid pBGKA

Plasmid pBGKA is derived from plasmid pBAD/GIII/A (Invitrogen) by replacement of the ampicillin resistance cassette through the kanamycin resistance cassette from plasmid pBHR1 [14].

pBAD/gIII system

pBAD/gIII plasmids are expression vectors that provide regulated expression of recombinant protein and purification in *E. coli*.
pBAD/gIII allows the secretion of expressed proteins into the periplasmic space where oxidative conditions favor the formation of structural disulfide bonds for the production of functional proteins. Periplasmic secretion also separates the recombinant protein from cytosolic proteases. The leader peptide for secreted expression into the periplasm originates from the bacteriophage fd gene III (gIII) protein. pBAD/gIII vectors contain also a C-terminal c-*myc* epitope for detection and analysis by Anti-*myc* antibodies, a C-terminal 6xHis Tag for rapid purification and detection with Anti-His (C-term) antibodies and an *araBAD* promoter for a tightly regulated expression [49] (see Fig. 8).

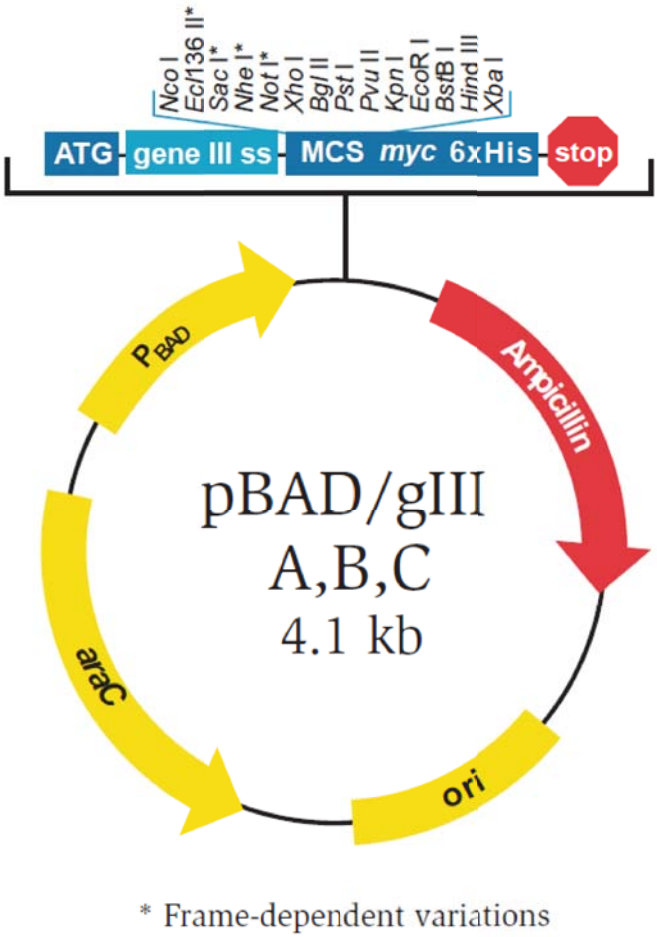


***Fig. 8: pBAD/gIII periplasmic expression system**, Invitrogen [49].*

araBAD promoter system

The araBAD promotor system allows tightly regulated expression as well as a dose-dependent induction and high protein yields.
The following features are included in all pBAD vectors:

- araBAD promotor for dose-dependent regulation
- araC gene for tight control of the araBAD promotor
- optimized ribosome binding site for increased translation efficiency
- rrnB transcription termination region for efficient transcript processing

The araBAD promotor is positively and negatively regulated by the product of the araC gene, a transciptional regulator. In the absence of arabinose, the AraC dimer

binds to the DNA and represses the expression by the formation of a 210 bp DNA loop. If arabinose is available it binds to AraC and releases the DNA loop which therefore allows the transcription to begin (see Fig. 9). Very low uninduced levels of transcription from the araBAD promotor are repressed by glucose. Glucose acts through lowering cAMP levels causing cAMP activator protein binding to the CAP site on the DNA and therefore lowering the stimulation of transcription [49].

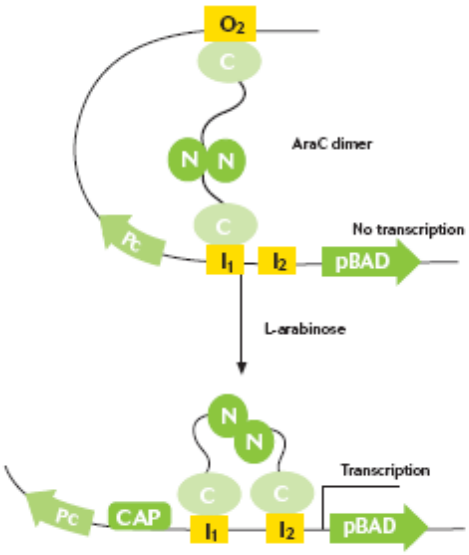


Fig. 9: Regulation of the araBAD promotor. *No transcription – AraC dimer binds to the DNA and leads to formation of a 210 bp DNA-loop. Transcription – L-arabinose binds to AraC and releases the DNA loop [49].*

3 Materials and methods

3.1 Bacterial strains, plasmids, media, antibiotics, cultivation

3.1.1 Bacterial strains

- *Escherichia coli* K12 NM522 sup E thi-1Δ(Lac-proAB)Δ(mcrB-hsdSM) 5(rK-mK-) (F'proABlacIqZΔM15) (Source: Stratagene, Heidelberg,Germany)
- *Escherichia coli* K12 C2988J (NEB 5-alpha competent *E.coli*) *fhuA2* Δ(*argF-lacZ)U169 phoA glnV44 Φ80*Δ (*lacZ*)M15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17* (Source: New England Biolabs, Germany)
- *Escherichia coli* K12 W3110 lon$^-$ lambda$^-$ F$^-$ mcrA mcrB IN(rrnD – rrnE) 1; (Source: Lab. Stock)

3.1.2 Plasmids

- Backbone plasmids

Name	Size	Resistance	Origin	Reference	Features
pBGKA	4040 bp	Kanamycin	pBR322	[14]	P_{BAD} / gIII-myc
pDRIVE-hCG-ß-LTB	4530 bp	Kanamycin / Ampicillin	pMB1	[51]	hCGß-LTB / P_{lac}
pMal-p2X	6721 bp	Ampicillin	pMB1	NewEngland Biolabs *Inc.*	LacIq / P_{tac}

- Expression plasmids

Name	Size	Resistance	Origin	Ref.	Features
pBGK-CGL	4763 bp	Kanamycin	pBR322	this work	gIII-hCGß-LTB-myc / P_{BAD}
pBAD/GIII/calmodulin	4556 bp	Ampicillin	pBR322	Invitrogen	P_{BAD} / gIII-calmodulin

- Lysis plasmids

Name	Size	Resistance	Origin	Reference	Features
pGLysivb	6201 bp	Gentamycin	Rep	[32]	λP_{Rmut}/Eivb
pGLysivb-2x	7168 bp	Gentamycin	Rep	this work	λP_{Rmut} / Eivb-Eivb
pGLMivb	6680 bp	Gentamycin	Rep	this work	LacIq / Eivb
pGULMivb	6696 bp	Gentamycin	Rep	this work	Uptake seq / LacIq / Eivb

3.1.3 Cultivation media

- Low salt LBv-Medium - 10g/L Peptone of Soya, 5g/L yeast extract and 5g/L NaCl, adjusted to a pH of 7.4.
- Plate - Count agar - plates for determination of the cfu – 23,5 g/L
- Low salt LB-Agar - 10g/L Peptone, 5g/L yeast extract and 5g/L NaCl, adjusted to a pH of 7.4. For agar plates 15 g agar/L is added to the media.

All media are autoclaved for 30 minutes at 121°C. All chemicals are purchased from Carl ROTH (Karlsruhe, Germany).

3.1.4 Antibiotics

Name	Stockconc. [mg/ml]	Endconc. [µg/ml]	µl / 5ml
Ampicillin	50	100	10
Kanamycin	25	50	10
Gentamycin	10	20	10
Tetracyclin	5	10	10
Streptomycin	20	100	25

3.1.5 Cultivation of *Escherichia coli*

Bacterial cultures are incubated in 5 ml LBv-medium in standard epprouvettes. For specific plasmids the corresponding antibiotics and/or additional glucose have to be added. The incubation is carried out at 36 ° C in a rotating incubator wheel (Heraeus BK5060E). The plates are incubated in a temperature regulating incubator at 28 ° C or 36 ° C (Heraeus BK5060E).

3.2 Buffers and solutions

50% Glycerin

25 ml 100% glycerin
25 ml dH_2O
mix well and sterilize by autoclaving

Agarose gel – 1% or 2%

weigh out 3 g or 6 g agarose
dissolve in 300 ml 1xTAE
melt in microwave and cool down with stirring to hand-hot
pour into electrophorese tray

0,85% Saline Medium

8,5 g NaCl
fill up with dH_2O to 1 liter
fill into epprouvettes 9 ml or 9,9 ml using dispenser
autoclave

GelRed staining solution

GelRedTM nucleic acid gel stain, 10000X in water (No. 41003) is ordered from Biotium and stored at RT protected from light.
15 µl GelRed stain
5 ml 1M NaCl
45 ml dH_2O
keep the staining solution protected from light

MOPS I

10,47 g MOPS (100mM)
0,74 g $CaCl_2$ x 2 H_2O (10mM)
0,6 g $RbCl_2$ (10mM)
dissolve in 400 ml dH_2O
adjust pH to 7,0 with KOH
fill up to 500 ml and autoclave

MOPS II

10,47 g MOPS (100mM)
5,15 g $CaCl_2$ x 2 H_2O (70 mM)
0,6 g $RbCl_2$ (10mM)
dissolve in 400 ml dH_2O
adjust pH to 6,5 with KOH
fill up to 500 ml and autoclave

PonceauS

0,2 g PonceauS
3,0 g Trichloric acetic acid
fill up to 100 ml dH_2O
it is reuseable

1xNuPage® Sample Buffer

6,5 ml PBS
2,5 ml NuPage® LDS Sample Buffer (4x)
1 ml NuPage® Reducing Agent (10x)

10x PBS

137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
2 mM KH_2PO_4
800 mL dH_2O
pH 7,4 with HCl
fill up to 1 L with dH_2O

50% Glucose - Dissolve Glucose Anhydrat = 50 g Glucose in 70 ml dH_2O and fill up to 100 ml
or
- dissolve Glucose Monohydrat = 55 g Glucose in 70 ml dH_2O and fill up to 100 ml
Filter sterilize or autoclave

3.3 Chemicals

Buffers, chemicals, media and solutions are purchased from:

Carl ROTH (Karlsruhe, Germany)
Merck (Darmstadt, Germany)

Materials for SDS-PAGE, Western blot and protein quantification are purchased from:

Invitrogen (Paisley, UK)
Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Carl Roth (Karlsruhe, Germany)

The markers are purchased from:

Fermentas Life Sciences (St. Leon-Rot, Germany)

3.4 Enzymes

Restriction and ligation enzymes are purchased from:

Fermentas Life Sciences (St. Leon-Rot, Germany)
New England Biolabs (Frankfurt a. Main, Germany)
Promega (Mannheim, Germany)

All reactions are performed according to the manufacturer's instructions.

3.5 Microbiological, molecularbiological and biochemical methods

3.5.1 Preservation of bacterial strains

Add 900 µl 50% sterile glycerins to the labeled cryo-tubes
Add 900 µl bacterial cultures
Mix by shaking
Stored by – 80°C

3.5.2 $CaCl_2$ / $RbCl_2$ competent cells

30 ml of sterile LBv-medium is inoculated with 1 ml over night bacterial culture. The culture is incubated under continuous shaking at 36°C till an OD_{600} of ~ 0,5 is reached. The culture is centrifuged for 10 minutes at 4 ° C and 4000 rpm. Thereafter, the supernatant is decanted, the pellet resuspended in 6 ml MOPS I and placed on ice for 10 minutes. The cells are again centrifuged for 10 minutes at 4 ° C and 4000 rpm. The supernatant is decanted and the pellet resuspended in 6 ml MOPS II and placed on ice for 30 minutes. The cells are centrifuged for 10 minutes at 4 ° C and 4000 rpm. The supernatant is decanted and the pellet is resuspended in 480 µl MOPS II and 180 µl of 50% glycerol and placed on ice for 10 minutes. Aliquots are made in 100 µl portions and frozen at -80 ° C.

The competent cells are checked by single cell strikes on LBv plates and by microscopy.

3.5.3 Transformation of $CaCl_2$ / $RbCl_2$ competent cells

The DNA (2 µl of Miniprep or 10-20 µl of ligation) is pipeted into the 100 µl aliquot of competent cells and placed for 30 minutes on ice. After that, a heat shock at 36 ° C or 42 ° C is performed for 2 minutes (depending of the plasmid used). The cells are placed for 5 minutes on ice. After addition of 700 µl LBv-medium, the cells are incubated on the shaker at 36 ° C. After the regeneration, the transformed cells are plated on agar plates with the coresponding antibiotics (100 µl and the remaining

rest). The agar plates are incubated over night at 36 ° C. Bacteria that have incorporated the plasmid can grow on the antibiotic-plates.

3.5.4 Preparation of plasmid DNA - Miniprep

The PeqLab Miniprep Kit I (PeqLab, Erlangen, Germany) is used according to the manufacturer's instructions. This PeqLab Miniprep Kit I is meant for 1-5 ml over night culture.

3.5.5 Preparation of plasmid DNA – Midiprep

- The PeqLab Midiprep Kit II (PeqLab, Erlangen, Germany) is used according to the manufacturer's instructions. This PeqLab Midiprep Kit II is meant for 50 ml over night culture.
- The Promega Midiprep Kit (Promega, Mannheim, Germany) is used according to the manufacturer's instructions. This Promega Midiprep Kit is meant for 100 ml over night culture.

3.5.6 Enzymatic reactions

3.5.6.1 Sequence specific cleavage of DNA

All restrictions digests are prepared according to the manufacturer's instructions.

3.5.6.2 Ligation

Ligation reactions are performed with T4 DNA Ligase and T4 ligase buffer (from New England Biolabs, Frankfurt a. Main, Germany) overnight at 4°C.

Ligation-mix	6 µl Insert
	3 µl Vector
	2 µl 10mM ATP
	2 µl T4 DNA ligase buffer (10x)
	1 µl T4 DNA ligase (5 u/µl)
	6 µl dd H_2O

3.5.7 Electrophoresis

DNA fragments are separated on 1% and 2% agarose gels (Maniatis *et al*., 1982) with 140 – 160 V and 1xTAE as running buffer. To determine the size of the fragments also a DNA marker is loaded (Fermentas GeneRuler™ 1kb marker, 50 bp marker). GelRed staining of the gel and observation under UV-light (250 nm) makes the DNA fragments visible. Gels are documented using Quantity One software (BioRad, Hercules, CA, USA).

3.5.8 Isolation and purification of DNA fragments

Isolations and purifications are performed with

- Clean and Concentrator Kit (GeneXpress, Wiener Neudorf, Austria)
- Gel Extraction Kit from Invitrogen (Invitrogen, Paisley, UK)
- PCR Purification Kit from Invitrogen (Invitrogen, Paisley, UK)

according to the manufacturer's instructions.

3.5.9 PCR

PCR reactions are performed using the iCycler (system) (Bio-rad, stadt, staat), according to the manufacturer's instructions. PCR enzymes used are purchased from Fermentas Life Sciences (St. Leon-Germany):

- Pfu DNA Polymerase (2,5 u/µl) + 10x Pfu buffer + $MgSO_4$
- Dream Taq DNA Polymerase (5 u/µl) + 10x Dream Taq buffer + $MgSO_4$

3.5.9.1 Test – PCR small scale

The PCR materials are thawed on ice.
Preparation of mastermix (100 µl):

- Polymerase (Pfu or Dream Taq) 1 µl
- 10x Buffer (Pfu/Dream Taq) + $MgSO_4$ 10 µl
- dNTPs (2mM) 10 µl
- Primer 1 (50 pmol/µl) 1 µl
- Primer 2 (50 pmol/µl) 1 µl
- ddH_2O 69 µl

Divide the mastermix into 3 labelled PCR eppis (each 23 µl)

- Eppi 1 23 µl Mastermix + 2 µl ddH_2O (negative control)
- Eppi 2 23 µl Mastermix + 1 µl template DNA + 1 µl ddH_2O
- Eppi 3 23 µl Mastermix + 2 µl template DNA

Put all 3 eppis into the PCR machine and start the PCR program

- Step 1 – (1 cycle)
  - Denaturation - 95°C 3 min
- Step 2 – (25 - 30 cycles)
  - Denaturation - 95°C 30 sec
  - Annealing - 48°C-60°C 30 sec (dependent on primer Tm)
  - Elongation - 72°C 1 min / 1000 bp product
- Step 3 – (1 cycle)
  - Final elongation - 72°C 10 min

3.5.9.2 PCR large scale

The PCR materials are thawed on ice.

Preparation of mastermix (300 µl):

- Polymerase (Pfu or Dream Taq) 3 µl
- 10x Buffer (Pfu/Dream Taq) + $MgSO_4$ 30 µl
- dNTPs (2mM) 30 µl
- Primer 1 (50 pmol/µl) 3 µl
- Primer 2 (50 pmol/µl) 3 µl
- ddH_2O 207 µl

For control divide the mastermix into 3 labelled PCR eppis (each 23 µl)

- Eppi 1 23 µl Mastermix + 2 µl ddH_2O (negative control)
- Eppi 2 23 µl Mastermix + 1 µl template DNA + 1 µl ddH_2O
- Eppi 3 23 µl Mastermix + 2 µl template DNA

For production divide the mastermix into 4 labelled PCR eppis (each 46 µl)

- Eppi 4 46 µl Mastermix + 4 ml template DNA
- Eppi 5-7 46 µl Mastermix + 2µl template DNA + 2µl ddH_2O

Put all 7 eppis into the PCR machine and start the PCR program

- Step 1 – (1 cycle)
  - Denaturation - 95°C 3 min
- Step 2 – (25 - 30 cycles)
  - Denaturation - 95°C 30 sec
  - Annealing - 48°C-60°C 30 sec (dependent on primer Tm)
  - Elongation - 72°C 1 min / 1000 bp product
- Step 3 – (1 cycle)
  - Final elongation - 72°C 10 min

3.5.10 Primers

Primers are received from Microsynth (Microsynth, Balgach, Swizerland)

3.5.10.1 Cloning of pBGK-CGL

Primer-set-1:

Fwd Primer: [BglII]

hCG[BglII]fwd: 5` AATAG ATC TCC AAG GAC CCG CTT 3´

Tm=54°C

Rev Primer: [BstBI]

hCG[BstBI]rev: 5` AATT TCG AAA GTT TTC CAT ACT GAT TGC 3´

Tm=52°C

…. Restriction sequence

___ Binding region within the insert fragment

Primer-set-2 (higher specificity):

Fwd Primer-2: [BglII]

hCG[BglII]fwd2: 5` AATAG ATC TCC AAG GAC CCG CTT CGG 3´

Tm=66°C

Rev Primer-2: [BstBI]

hCG[BstBI]rev2: 5` AATT TCG AAA GTT TTC CAT ACT GAT TGC CGC A 3´

Tm=66°C

…. Restriction sequence

___ Binding region within the insert fragment

3.5.10.2 Cloning of pGLysivb-2x

Fwd Primer: [NcoI]

Eivb(NcoI)fwd: 5` AAT CCA TGG TCA GCC AAA CGT CTC TTC 3´

Tm=54°C

Rev Primer: [XhoI]

Eivb(XhoI)rev: 5` AAT CTC GAG TCA TTC GTG CCA TTC GAT T 3´

Tm=56°C

…. Restriction sequence

___ Binding region within the insert fragment

3.5.10.3 Cloning of pGLMivb

Fwd Primer: [XhoI]

LacIq-XhoI-fwd: 5` ATA CTC GAG CAC CAT CGA ATG GTG CAA A 3´

Tm=56°C

Rev Primer: [SbfI]

LacIq-SbfI-rev: 5` ATA CCT GCA GGA CTG GCT GTT TCC TGT 3´

Tm=56°C

…. Restriction sequence

___ Binding region within the insert fragment

3.5.10.4 Cloning of pGULMivb

Fwd Primer: [XhoI]

LacIq-XhoI-fwd-2: 5` ATA CTC GAG CCG TCT GAA ATT CCG ACA CCA TCG AAT G 3´

Tm = 64°C

Rev Primer: [SbfI]

LacIq-SbfI-rev: 5` ATA CCT GCA GGA CTG GCT GTT TCC TGT 3´

Tm=56°C

5'GCCGTCTGAA 3' 10-Base-Pair sequence for Uptake of DNA

…. Restriction sequence

___ Binding region within the insert fragment

3.6 Protein gel electrophoresis and Western blot

3.6.1 Protein sample preparation of bacterial culture (before lysis)

The protein samples (1 ml) are diluted according to the OD_{600}:

OD_{600} value x 250 = Volume of 1x sample buffer

The sample pellet is resuspended in the 1x sample buffer and it is mixed well. After that it is incubated at 99°C for 10 minutes. The protein sample can be stored at -20°C or used directly. Before loading on the gel the samples have to be spined down for 3 minutes at maximum speed. The supernatants (~15 µl) are loaded on the gel.

3.6.2 Protein sample preparation of lysed culture (after lysis)

The protein samples (1 ml) are diluted according to the OD_{600}:

OD_{600} value (highest value before lysis) x 250 = Volume of 1x sample buffer

The sample pellets of lysed cultures are resuspended always in the highest calculated volume of 1x sample buffer before lysis. Further treatment is done as for the samples before lysis.

3.6.3 Protein sample preparation of lyophilized BG

10 mg of lyophilized BG are weight and resuspended in 1 ml ddH_2O. 200 µl of the BG suspension is mixed with 200 µl 1x NuPage sample buffer and incubated at 99°C for 10 minutes. The suspension is spined down for 3 minutes at maximum speed. 100 µl of the supernatant are diluted with 400 µl 1x NuPage sample buffer. 2 µl, 5 µl 10 µl and 20 µl of the diluted sample are transferred in extra eppis and filled up with 1x NuPage sample buffer to a total volumen of 20 µl. The whole volume (20 µl) is loaded on the gel.

3.6.4 Protein sample preparation of positope (positive control) for quantification

The positope (Invitrogen) with a molecular weight of 53 kDa is delivered as solution with a concentration of 25 ng/µl. A serial dilution (1:2) of positope is made:

Standard	Serial dilution – 1:2	Final concentration
1.	40 µl of positope	500 ng/20µl
2.	40 µl of positope + 40 µl of 1x sample buffer	250 ng/20µl
3.	40 µl of 2. standard + 40 µl of 1x sample buffer	125 ng/20µl
4.	40 µl of 3. standard + 40 µl of 1x sample buffer	62,5 ng/20µl
5.	40 µl of 4. standard + 40 µl of 1x sample buffer	31,25 ng/20µl

The standard samples are incubated at 99°C for 10 minutes and loaded on the gel (20 µl) without spin down.

3.6.5 NuPAGE® Bis-Tris Electrophoresis system

The proteins are separated using the NuPage® Bis-Tris Electrophoresis System (Invitrogen, Paisley, UK) according to the manufacturer's instructons.

15 µl or 20 µl of prepared protein samples (see above) and 5 µl of molecular weight protein marker are loaded on gel, run at 180 Volt for 60 minutes.

3.6.6 Western blot

The protein transfer to nitrocellulose membrane is carried out using an xCell II Blot Module (Invitrogen, Paisley, UK) according to the manufacturer's instructons. The blotting conditions are 30 Volt constant for blotting one gel or 60 Volt constant for blotting two gels for 60 minutes.
Transfer efficiency and location of the protein marker is checked by staining with Ponceau S.
Membrane blocking is carried out at 4°C overnight using Blocking Solution (1x Roti-Block™, Carl Roth, Karlsruhe, Germany).

After washing 3 x 5 minutes and 1 x 10 minutes in TBST the membrane is incubated with the HRP-coupled first antibody for 1 hour. After antibody incubation the membrane is washed 3 x 5 minutes and 1 x 10 minutes in TBS. Membranes are developed with 3ml ECL-chemiluminescent reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to manufacturer's instructions.
The Quantity One software (BioRad, Hercules, CA, USA) is used for the documentation and quantification.

List of used antibodies:

Direct detection antibodies	Species type	Uses	Dilution	Recognizes	Source
Anti-myc-HRP	mouse monoclonal IgG antibody	WB	1 to 5.000 in TBS	recombinant proteins containing the c-myc epitope	Invitrogen
Anti-His(C-term)-HRP	mouse monoclonal IgG antibody	WB	1 to 5.000 in TBS	polyhistidine amino acid sequence at the C-terminus of a protein	Invitrogen

3.7 Growth, Expression and Lysis of *Escherichia coli*

3.7.1 Growth study

In the growth study the optimal growth temperature is determined for a bacterium. This experiment is carried out in 100 ml noseflasks with 25 ml of LBv. The growth of bacteria at different temperatures is observed. Inoculation is performed at OD_{600} ~ 0.1 and growth observed for 240 minutes. At regular intervals OD, cfu and microscopy samples are taken, as seen from the following growth template.

1. Growth- Template:

		Bacterium Strain	Bacterium Strain	Bacterium Strain	Bacterium Strain	Bacterium Strain	Bacterium Strain			
		1	2	3	4	5	6			
		28°C	28°C	36°C	36°C	42°C	42°C			
Min:	Nr.	25 ml LBv	25 ml LBv	25 ml LBv	25 ml LBv	25 ml LBv	25 ml LBv	OD	CFU each 2x50 µl	Micro scope
0	**inoc**							1-6		
30	**A**							1-6	1-6	
60	**B**							1-6	1-6	1,3,6
120	**C**							1-6	1-6	
180	**D**							1-6	1-6	
240	**E**							1-6	1-6	1,3,6

2. Growth- Template:

		Bacterium Strain	Bacterium Strain	Bacterium Strain	Bacterium Strain	Bacterium Strain	Bacterium Strain			
		7	8	9	10	11	12			
		40°C	40°C	42°C	42°C	44°C	44°C			
Min:	Nr.	25 ml LBv	25 ml LBv	25 ml LBv	25 ml LBv	25 ml LBv	25 ml LBv	OD	CFU each 2x50 µl	Micro scope
0	**inoc**							1-6		
30	**A**							1-6	1-6	
60	**B**							1-6	1-6	1,3,6
120	**C**							1-6	1-6	
180	**D**							1-6	1-6	
240	**E**							1-6	1-6	1,3,6

3.7.2 **Lysis control study**

In the lysis control study one positive clone of bacterial strain with lysis plasmid is compared with a control strain with backbone plasmid in lysis behaviour. Experiments are carried out in the nose flasks with 25 ml of LBv and the corresponding antibiotics. For inoculation (OD_{600} ~ 0.1) the overnight culture of *E. coli* (lysis plasmid) and *E. coli* (backbone plasmid) are used. The nose flasks are incubated in a water bath at 36 ° C and 350 rpm. At OD_{600} ~ 0.4 lysis is induced by temperature upshift to 42 ° C and observed for the next 180 minutes. Uninduced samples are included as negative controls. During observation time OD, cfu, microscopy and western blot samples are taken (see Lysis control template).

Lysis control template:

				Bacterium Strain (backbone plasmid)	Bacterium Strain (backbone plasmid)	Bacterium Strain (lysis plasmid)	Bacterium Strain (lysis plasmid)				
				1	**2**	**3**	**4**				
Time	Min	OD	Nr.	25 ml LBv + AB 36°C	25 ml LBv + AB 36°C 42°C at B	25 ml LBv + AB 36°C	25 ml LBv + AB 36°C 42°C at B	OD	CFU 1 x 50µl 1 x 100µl	WB 1ml	Micro scope
		0,1-0,15						1-4			
								1-4			
		0,2 - 0,25	**A**					1-4	1-4		1-4
	0	0,3 - 0,4	**B**					1-4	1-4	1-4	
	20		**C**					1-4	2+4	2+4	2+4
	40		**D**					1-4	2+4	2+4	2+4
	60		**E**					1-4	1-4	1-4	1-4
	90		**F**					1-4	2+4	2+4	2+4
	120		**G**					1-4	1-4	1-4	1-4
	180		**H**					1-4	1-4	1-4	1-4

3.7.3 Growth- and Lysis- study in small scale

Postive clones of bacterial strain with lysis plasmid (always in triplets) are checked for a Lysis consistency study to determine the clone with the best lysis behaviour. Experiments are carried out in the nose flasks with 25 ml of LBv and the corresponding antibiotics. For inoculation (OD_{600} ~ 0.1) the overnight culture of *E. coli* (lysis plasmid) is used. The nose flasks are incubated in a water bath at 36 ° C and 350 rpm. At OD_{600} ~ 0.5 lysis is induced by temperature upshift to 42 ° C and observed for the next 120 minutes. During observation time OD, cfu, microscopy and western blot samples are taken (see Lysis template).

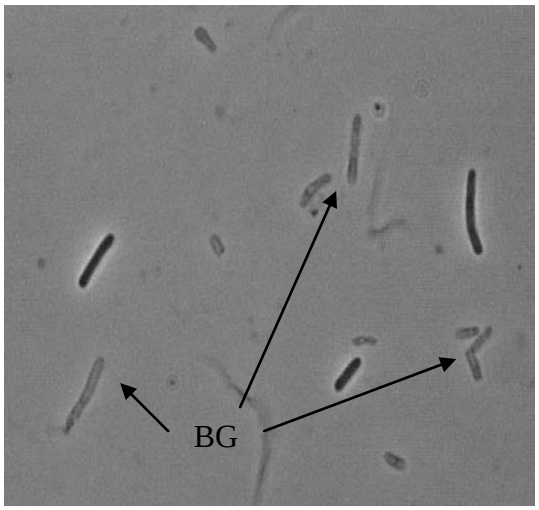
Lysis- Template:

			Strain Plasmid	Strain Plasmid	Strain Plasmid				
			clone 1	clone 2	clone 3				
			1	2	3				
Min:	OD	Nr.	25 ml LBv + AB 36°C 42°C at B	25 ml LBv + AB 36°C 42°C at B	25 ml LBv + AB 36°C 42°C at B	OD	CFU each 4x50µl	West. blot 1ml	Micros cope
	0,1-0,15					1-3			
						1-3			
	0,25 -0,3	**A**				1-3	1-3		1-3
0	0,4 - 0,5	**B**				1-3	1-3	1-3	
10		**C**				1-3	1-3	1-3	
20		**D**				1-3	1-3	1-3	
40		**E**				1-3	1-3	1-3	
60		**F**				1-3	1-3	1-3	1-3
90		**G**				1-3	1-3	1-3	
120		**H**				1-3	1-3	1-3	

3.7.3.1 Microskopie

Bacterial Ghosts are distinguished from living bacteria. BG's are much brighter than the living bacteria.

BG's of *E. coli* K12 NM522 (pGLysivb):



3.7.3.2 Determination of live cell counts, colony forming units = cfu

The viable cell counts (CFU) are determined using a spiral plater (Don Whitley Scientific Limited, West Yorkshire, UK). Before plating the bacterial samples are serially diluted in saline (0,85% NaCl). For 1:10 dilutions 9 ml saline epprouvettes (9 ml saline + 1 ml culture) and for 1:100 dilution 9,9 ml saline epprouvettes (9,9 ml saline + 0,1 ml culture) are used.
Following dilutions according to the OD_{600} of growing and lysing cultures are given as a reference:

Before lysis / Growth			After lysis – induction	
OD_{600}	Dilution		OD_{600}	Dilution
0,05 – 0,5	10^4		1,5 – 1,0	10^5
0,5 – 1,0	10^5		1,0 – 0,5	10^4
1,0 – 2,0	10^6		0,5 – 0,2	10^3
			0,2 – 0,01	10^2

50 and/or 100 µl of the final dilutions are plated in logarithmic manner on count agar plates without any antibiotics. The plates are incubated at 28°C or 36°C over night and afterwords counted in the Synbiosis ProtoCOL Colony Counter machine (3.15, Synoptics Ltd., Cambridge, UK).

Cfu values are illustrated as a curve in a logarithmic manner together with the OD_{600} values in one curve.

3.7.4 Protein Expression- Study in small scale

Expression study template:

			E.coli (pBGKA) negative contr.	*E.coli* (pBAD/GIII/ Calmodulin) positive contr.	*E.coli* (Expression plasmid)	
			1	2	3	West.
Min:	OD	Nr.	25 ml LBv 36°C	25 ml LBv 36°C	25 ml LBv 36°C	blot 1ml
	0,1					
	0,2-0,3					
0	0,4-0,5	A				1-3
30		B				1-3
60		C				1-3

Experiments are carried out in the nose flasks with 25 ml of LBv and the corresponding antibiotics. For inoculation (OD_{600} ~ 0.1), the overnight cultures of *E. coli* (expression plasmid) are used.The nose flasks are incubated in a water bath at 36 ° C and 350 rpm. The protein expression is induced at OD_{600} ~ 0.4-0.5 by addition of L-arabinose (final concentration 0.2%). Expression is carried out for the next 60 minutes, during the entire study samples for western blot are taken (see expression study template).

3.7.5 Expression- and Lysis- Study in small scale

Experiments are carried out in nose flasks with 25 ml LBv and the corresponding antibiotic. For inoculation (OD_{600} ~ 0.1), overnight culture of *E. coli* (expression plasmid + lysis plasmid) are used. The nose flasks are incubated in a water bath at 36 ° C and 350 rpm. The protein expression is induced at OD_{600} ~ 0.25 by addition of L-arabinose (final concentration 0,2%) and further incubated at 36 ° C. The lysis is induced at OD_{600} ~ 0.5 by temperature upshift to 42 ° C. Lysis is carried out for the

next 120 minutes. During the entire study OD, cfu, microscopy and Western blot samples are taken (see Expression and lysis-study template).

Expression- and Lysis- Study template:

			Strain Plasmid	Strain Plasmid	Strain Plasmid			
			clone 1	clone 2	clone 3			
			1	2	3			
Min:	OD	Nr.	25 ml LBv + AB 36°C 42°C at B	25 ml LBv + AB 36°C 42°C at B	25 ml LBv + AB 36°C 42°C at B	OD	CFU each 4x50µl	Microscope
	0,1-0,15					1-3		
						1-3		
	0,25 -0,3	**A**				1-3	1-3	1-3
0	0,4 - 0,5	**B**				1-3	1-3	
20		**C**				1-3	1-3	
40		**D**				1-3	1-3	
60		**E**				1-3	1-3	
90		**F**				1-3	1-3	1-3
120		**G**				1-3	1-3	
150		**H**				1-3	1-3	

3.8 Fermentation in large scale

For the production of BG in large scale 30 Liter fermentor – TECHFORS S (Infors AG, Bottmingen, Switzerland) is used (with a working volume of 20 Liter).

3.8.1 Media preparation

20 liters of LBv media are prepared and autoclaved (121°C) 18 hours before fermentation starts. During this time the IRIS program observs the pH, temperature, oxygen and flow. Before inoculation with the over night culture (~2 Liter) the sterility of the medium is controled by taking blank samples and plating them on count agar plates.

3.8.2 Overnight culture

Working stocks of the production clone (-80°C) are thawn and used for the inoculation of 4 x 500 ml LBv with corresponding antibiotics. These four flasks are incubated overnight in water bath at 35°C.

3.8.3 Fermentation process

At the begining of fermentation, before the fermentor is inoculated with the overnight culture, corresponding antibiotics are added. During fermentation different parameters (temperature, oxygen flow, pO2, pH, rpm) are observed via the IRIS program. In regular intervals 50 ml samples are taken to determine OD_{600}, cfu and for western blot and microscopy.
The protein expression is induced by addition of L-Arabinose (Final concentration 0,2%) at OD_{600} ~ 0,6.
The lysis induction is induced 60 minuten after protein induction by temperature upshift to 44°C.

3.8.4 Killing

90 minuten after lysis induction the non-lysed bacteria are killed by the addition of double concentration of tetracyclin and streptomycin. The killing is performed for next 60 minutes.

3.8.5 Harvesting

The BG's are harvested using large and small tangential flow filtration (TFF) modules.

3.8.6 Lyophilisation

The 200 ml final products are spread to 10 lyophilization flasks (10 * 20 ml). The collection bottle is rinsed with some additional 10 ml of dH_2O, the remainings are transferred to an eleventh lyophilization bottle. All lyophilization bottles are stored at -20°C, followed by storage at -80°C and lyophilized afterwards.

3.8.7 Calculation of Lysis efficacy and particles per mg

The lysis efficiency is calculed using the following equation:

$$\textit{Lysis efficacy [\%]} = [1 - (CFU_{lowest} / CFU_{highest})] \times 100\%$$

The particles per mg are calculated using the following equation:

$$\textit{Particles per mg } [mg^{-1}] = (cfu_{highest} \times volume_{total}) / weight_{lyophilized}$$

3.8.8 Sterility testing of the material

The sterility testings are prepared in triplets. For each set a 10 mg of lyophilized BG are resuspended in 1,5 ml LBv. 100 µl and 200 µl of the resuspension are plated on LBv plates. 1 ml of the resuspension is mixed with 20 ml hand warm LB agar in an empty agar plate. 100 µl of the resuspension are added to a 5 ml LBv epprouvette. Plates and epprouvette are incubated over night at 36°C. On the next day 100 µl and 200 µl of the liquid material are plated on LBv agar plates and incubated until the next day.

4 Results

4.1 Bacterial ghosts as carrier of human chorionic gonadotropin – ß fused to the heat labile enterotoxin of *E. coli* (hCG-ß-LTB)

The objective of my diploma thesis was the production of hCG-ß-LTB expression plasmids for BG - CV.

4.1.1 Construction of hCG-ß-LTB expression plasmid for BG - CV

4.1.1.1 Overview of the strategy

Plasmid pDrive-hCG-ß-LTB was received from Dr. Talwar (Talwar research foundation, New Delhi, India).

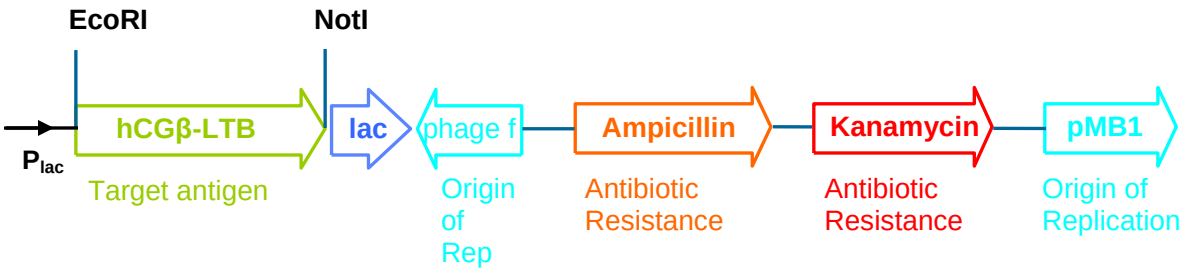


Fig. 10: Linear map of pDrive-hCG-ß-LTB *(Talwar research foundation, New Delhi, India) Contains target antigen hCG-ß-LTB between restriction sites of EcoRI and NotI.*

Plasmid pDrive hCG-ß-LTB (4530bp) contains the immunogenic sequence hCG-ß-LTB (760bp) between restrictions sites of EcoRI and NotI (see Fig. 10).

HCG-ß-LTB is a fusionprotein and consists of:

- hCGß (ß-subunit of human chorionic gonadotropin) sequence as antigen
- LTB (heat labil toxin subunit B of *E. coli*) sequence as adjuvant
- Linking-sequence (TCT AGA), a short sequence which connects hCGß- and LTB-sequence

The nucleotide sequence of hCG-ß-LTB is pictured in Tab. 1, the corresponding polypeptide sequence of hCG-ß and LTB is given in Tab. 2.

EcoRI

GAA TTC TCC AAG GAC CCG CTT CGG CCA CGG TGC CGC CCC ATC AAT GCC ACC
CTG GCT GTG GAG AAG GAG GGC TGC CCC GTG TGC ATC ACC GTC AAC ACC ACC
ATC TGT GCC GGC TAC TGC CCC ACC ATG ACC CGC GTG CTG CAG GGG GTC CTG
CCG GCC CTG CCT CAG GTG GTG TGC AAC TAC CGC GAT GTG CGC TTC GAG TCC
ATC CGG CTC CCT GGC TGC CCG CGC GGC GTG AAC CCC GTG GTC TCC TAC GCC
GTG GCT CTC AGC TGT CAA TGT GCA CTC TGC CGC CGC AGC ACC ACT GAC TGC
GGG GGT CCC AAG GAC CAC CCC TTG ACC TGT GAT GAC CCC CGC TTC CAG GAC
TCC TCT TCC TCA AAG GCC CCT CCC CCC AGC CTT CCA AGC CCA TCC CGA CTC CCG
GGG CCC TCG GAC ACC CCG ATC CTC CCA CAA **TCT AGA** GGA GCT CCT CAG TCT ATT
ACA GAA CTA TGT TCG GAA TAT CAC AAC ACA CAA ATA TAT ACG ATA AAT GAC AAG
ATA CTA TCA TAT ACG GAA TCG ATG GCA GGC AAA AGA GAA ATG GTT ATC ATT ACA
TTT AAG AGC GGC GCA ACA TTT CAG GTC GAA GTC CCG GGC AGT CAA CAT ATA
GAC TCC CAA AAA AAA GCC ATT GAA AGG ATG AAG GAC ACA TTA AGA ATC ACA
TAT CTG ACC GAG ACC AAA ATT GAT AAA TTA TGT GTA TGG AAT AAA ACC CCC AAT
TCA ATT GCG GCA ATC AGT ATG GAA AAC TAG **GCG GCC GC**

Not I

Tab. 1: Nucleotide sequence of hCG-ß-LTB gene. *The green colored sequence represents the hCG-ß gene and the red colored sequence the LTB gene. The sequence was cloned into the cloning vector pDrive (Invitrogen, Paisley, UK) with restrictions sites marked as above.*

Amino Acid Sequence of hCG-ß:

SKDPLRPRCRPINATLAVEKEGCPVCITVNTTICAGYCPTMTRVLQGVLPALPQVV CNYRDVRFESIRLPGCPRGVNPVVSYAVALSCQCALCRRSTTDCGGPKDHPLTCD DPRFQDSSSSKAPPPSLPSPSRLPGPSDTPILPQ

Amino Acid Sequence of LTB:

GAPQSITELCSEYHNTQIYTINDKILSYTESMAGKREMVIITFKSGATFQVEVPGSQH IDSQKKAIERMKDTLRITYLTETKIDKLCVWNKTPNSIAAISMEN

Tab. 2: Polypeptide sequence of hCGß and LTB gene

The plasmid pDrive hCG-ß-LTB was transformed into *E. coli K12* NM522 and glycerin stocks were produced. Before using the plasmid for cloning it was checked via restriction enzyme digests. Following restriction enzymes were used:

BglII, Bsp119I	---	no cut
NcoI, SacI, KpnI, EcoRI, XbaI	4530 bp	one cut
PstI	165 / 4365 bp	two cuts
PvuII	517 / 625 / 3388 bp	three cuts

All restriction enzymes cut as expected, except NcoI (see Fig. 11).

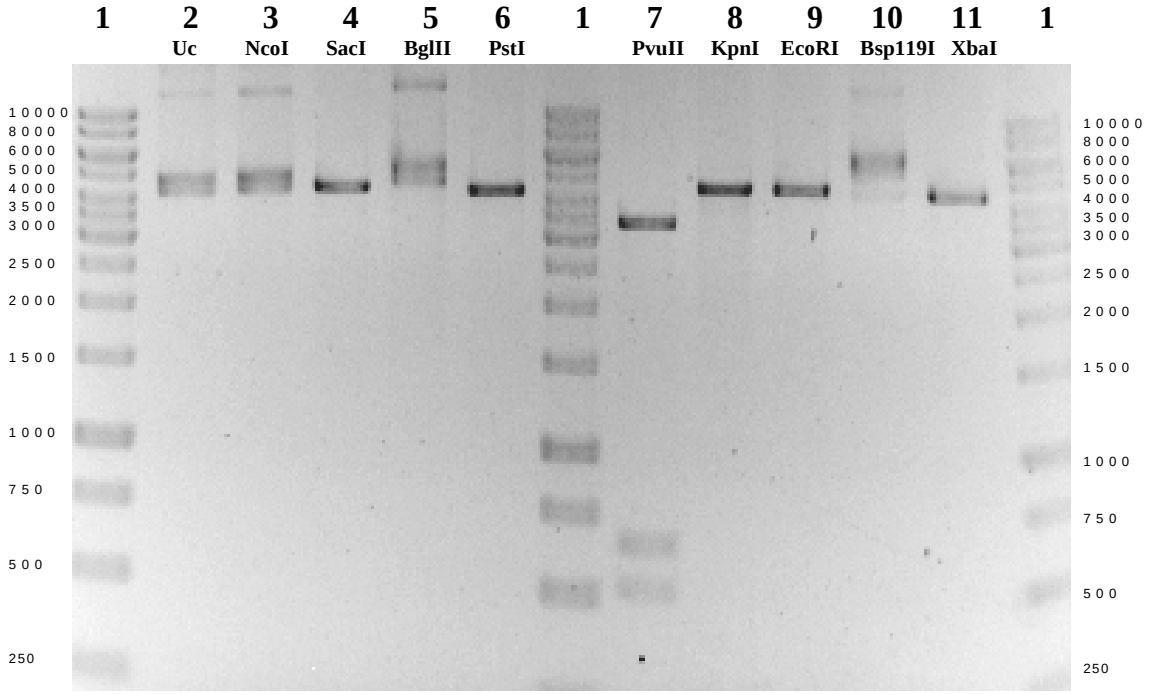


Fig. 11: Control-digest of pDrive-hCG-ß-LTB.

Lane 1 – Fermentas GeneRuler 1kb DNA ladder; lane 2 – uncut pDrive-hCGß-LTB - correct; lane 3 – NcoI, single cutter, no cut; lane 4, 8, 9, 11– cut with SacI, KpnI, EcoRI, XbaI, expected size – 4530bp - correct; lane 5, 10 – cut with BglII, Bsp119I, non cutter - correct; lane 6 – cut with PstI, expected size – 165/4365bp – correct (small fragment not visible); lane 7 – cut with PvuII, expected size – 517/625/3388bp - correct.

4.1.1.2 Cloning of pBGK-CGL plasmid

Plasmid pBGK-CGL was cloned using pBGKA as a backbone for hCG-ß-LTB. For cloning strategy see Fig. 12. pBGK-CGL plasmid carries the pBAD promoter, the GIII signal sequence for transport of the fusion protein to the PPS and a Kanamycin resistance cassette. Further the plasmid carries the gene for the hCG-ß fused to the LTB (heat labil toxin subunit).

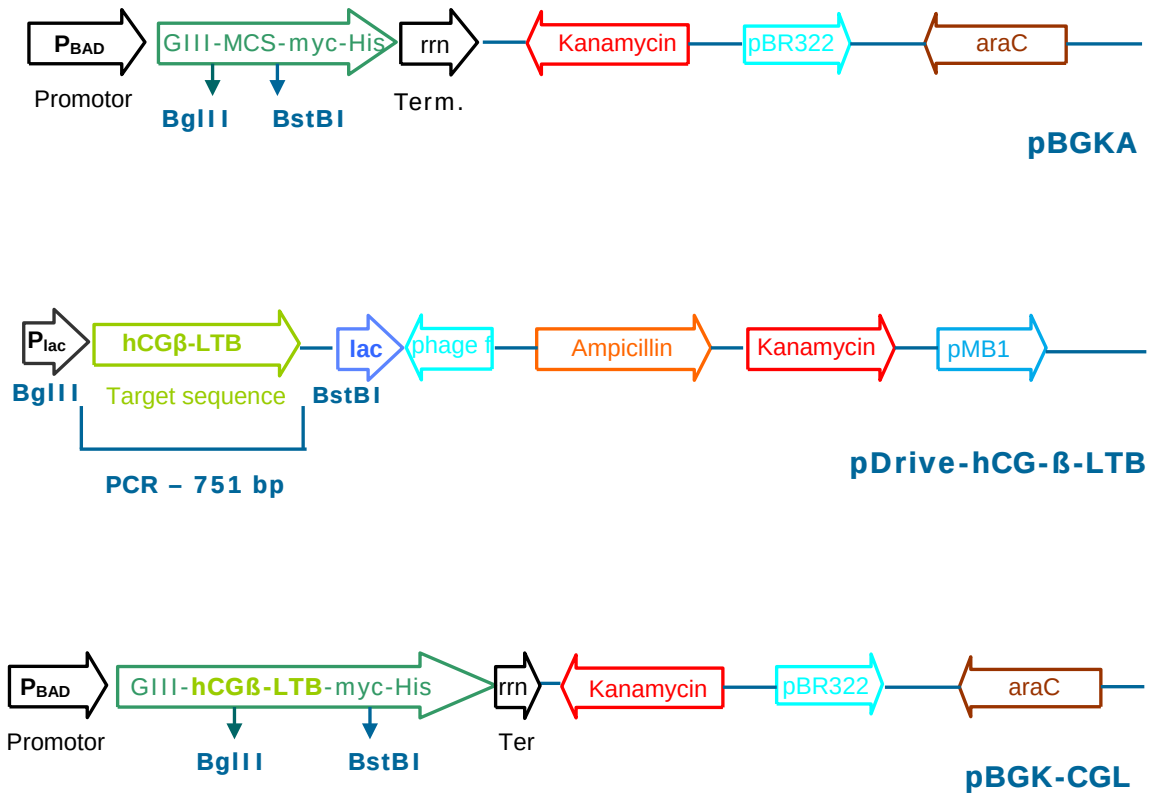


Fig. 12: Cloning strategy of pBGK-CGL. *pBGKA is the backbone plasmid. pDrive-hCGß-LTB is the donor plasmid with target sequence hCG-ß-LTB. pBGK-CGL is the new plasmid containing a construct of GIII-hCG-ß-LTB-myc-His under control of arabinose inducible promotor pBAD.*

- ***Insert production***

The Insert (hCG-ß-LTB) was amplified via PCR, from plasmid pDrive-hCG-ß-LTB. Specific primers with restriction sites for BglII or BstBI were designed. The PCR product is shown in Fig. 13.

Fwd Primer-2: [BglII]

hCG[BglII]fwd2: 5` AATAG ATC TCC AAG GAC CCG CTT CGG 3´

Tm=66°C

Rev. Primer-2: [BstBI]

hCG[BstBI]rev2: 5` AATT TCG AAA GTT TTC CAT ACT GAT TGC CGC A 3´

Tm=66°C

…. Restriction sequence

___ Binding region within the insert fragment

PCR was carried out according to standard protocol using:

- Dream taq-Polymerase (5 u/µl) (Fermentas)
- 10x dream-taq-buffer (+$MgCl_2$)
- dNTPs Mix (2mM)
- annealing temperature calculated according to primers melting temperature: 60°C
- elongation time: 60 sec / cyclus, 72°C

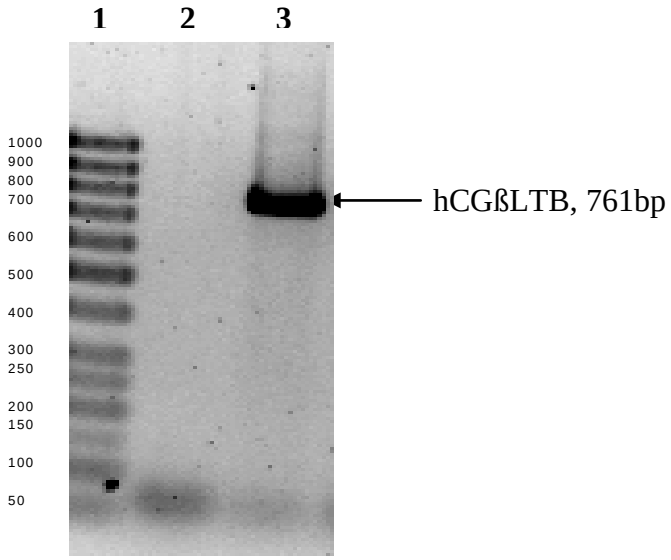


Fig. 13: hCG-ß-LTB PCR-product *on 2% agarose. Lane 1 - 50bp DNA leader (fermentas); lane 2 - negative control for PCR; lane 3: hCG-ß-LTB PCR-product (undigested) - 761bp - correct.*

The PCR-product with the expected size of 761 bp, was eluted from 1% agarose gel using Xact DNA gel extraction Kit (Genexpress). Subsequently the product was purified a second time with Xact DNA gel extraction Kit (Genexpress) and double-digested with BglII and BstBI = Bsp119I (conventional Enzymes from Fermentas). The digestion supplied a fragment with the size of 751bp which was further purified with Xact DNA gel extraction Kit (Genexpress), checked on a 1% agerose gel (see Fig. 14) and used for cloning into pBGK-CGL.

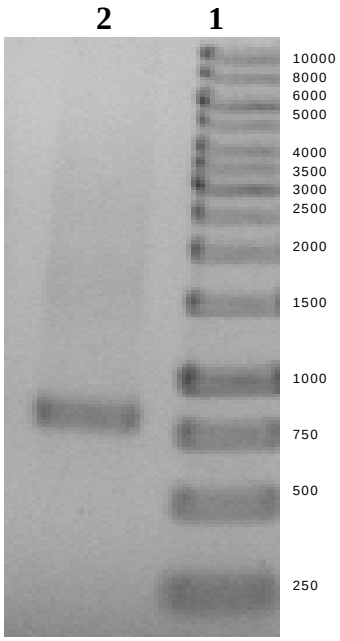


Fig. 14: Digested and purified hCG-ß-LTB PCR-product *(751 bp), on 1% agarose gel. Lane 1 - Fermentas GeneRuler 1kb DNA ladder; lane 2: hCG-ß-LTB - After digestion with BglII and Bsp119I and purification.*

- ***Vector production***

Plasmid pBGKA (Diploma thesis of Simone Schlacher, 2009) was used as backbone vector for cloning of pBGK-CGL.
Plasmid pBGKA (4040bp, see Fig. 12) was checked via digestion by several restriction enzymes (see Fig. 15). Afterwards it was also double-digested with BglII and BstBI = Bsp119I (conventional enzymes from Fermentas). The digestion supplied two fragments 4012bp and 28bp. The bigger fragment was purified with Xact DNA gel extraction Kit from Genexpress (see Fig. 16). To avoid any religation of the vector, the 4012bp fragment was dephosphorylated with BAP (bacterial alkaline Phosphatase from Fermentas).

Ligation of the vector fragment (4012bp) with the insert fragment (751bp) was performed at 4°C and over night, using T4 DNA ligase from NEB. After transformation into *E. coli* K12 C2988J, positive clones were identified by restriction analysis.

A. **B.**

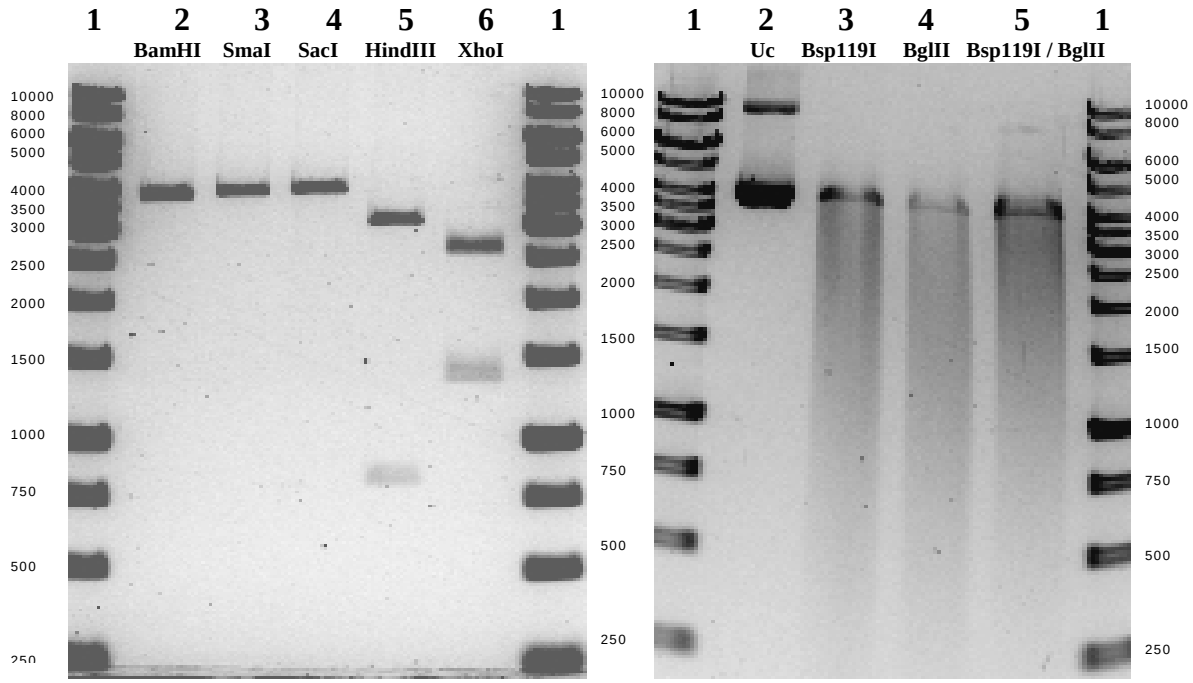


Fig. 15: Control-digest of pBGKA.

A: *fast digest enzymes from Fermentas → lane 1: Fermentas GeneRuler 1kb DNA ladder; lane2: BamHI 4040bp correct; lane3: SmaI 4040bp correct; lane4: SacI 4040bp correct; lane5: HindIII 829/3211bp correct; lane6: XhoI 1384/2656bp correct.*

B: *conventional enzymes from Fermentas → lane1: Fermentas GeneRuler 1kb DNA ladder; lane2: uncut pBGKA; lane3: Bsp119I 4040bp correct; lane4: BglII 4040bp correct; lane 5: double-digest Bsp119I / BglII 4012/28bp correct (small fragment not visible)*

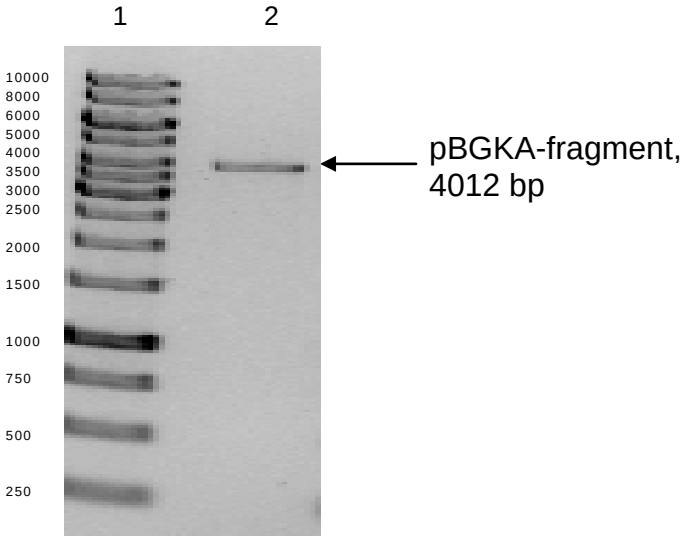


Fig. 16: Purified pBGKA-fragment *(4012 bp), on 1% agarose gel.*

Lane 1 - Fermentas GeneRuler 1kb DNA ladder; lane 2 - pBGKA-fragment (4012bp) – After double digestion with BglII / Bsp119I and purification.

Positive clones were control-digested with ApaI (one cut in insert – hCG-ß-LTB, see Fig. 17) and with SmaI (cuts in insert – 2x and in vector – 1x, see Fig. 17). Digest of pBGK-CGL with ApaI or SmaI results theoretically in:

ApaI	1 fragment	4763 bp
SmaI	3 fragments	3340 / 1231 / 192 bp

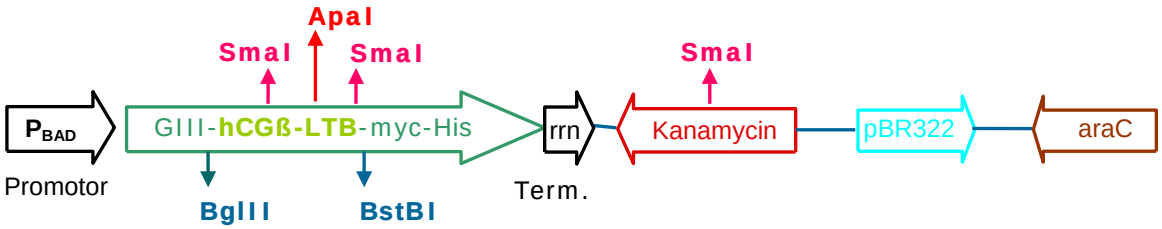


Fig. 17: Theoretical restriction pattern of pBGK-CGL.

ApaI cuts 1x in insert (= hCG-ß-LTB); SmaI cuts 2x in insert and 1x in vector (= pBGKA).

The control restriction analysis corresponded to the theoretical predicted size of pBGK-CGL (see Fig. 18). Positive clones were stored as glycerinstock and as midiprep.

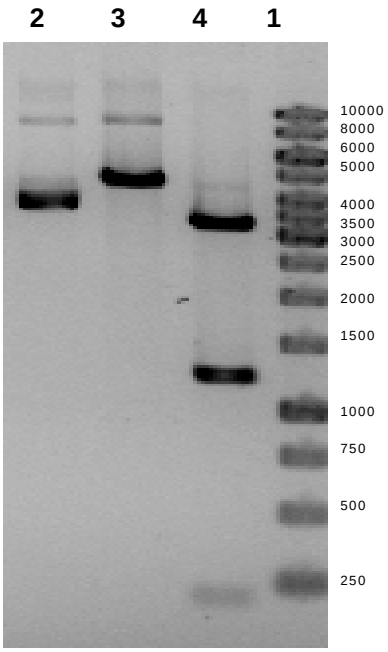


Fig. 18: Restriction analysis of correct pBGK-CGL clone.

Lane 1: Fermentas GeneRuler 1kb DNA ladder; lane 2: pBGK-CGL uncut; lane 3: ApaI 4763bp correct; lane 4: SmaI 3340/1231/192bp correct.

4.1.1.3 Protein expression study of hCG-ß-LTB antigen from pBGK-CGL

Via a protein expression study it is possible to test which pBGK-CGL clone shows the best expression of hCG-ß-LTB. In the same time it's important to control that the antigen has no negative influence on the bacterial growth.
The experiments were performed in 20 ml LBv with kanamycin with the following combinations:

- *E.coli* K12 C2988J (pBGKA) as negative expression-control for WB (pBAD/gIII system without recombinant protein)
- *E.coli* K12 C2988J (pBAD/GIII/calmodulin) as positive expression-control for WB (pBAD/gIII system with calmodulin as recombinant protein)
- *E.coli* K12 C2988J (pBGK-CGL) – expression of hCGß-LTB by positive clones

Optical density was messured at 600 nm. At OD_{600} ~ 0,3 the expression was induced by adding L-arabinose at a final concentration of 0,2%. The expression took 1h, during this time protein samples for expression-analyse were taken.

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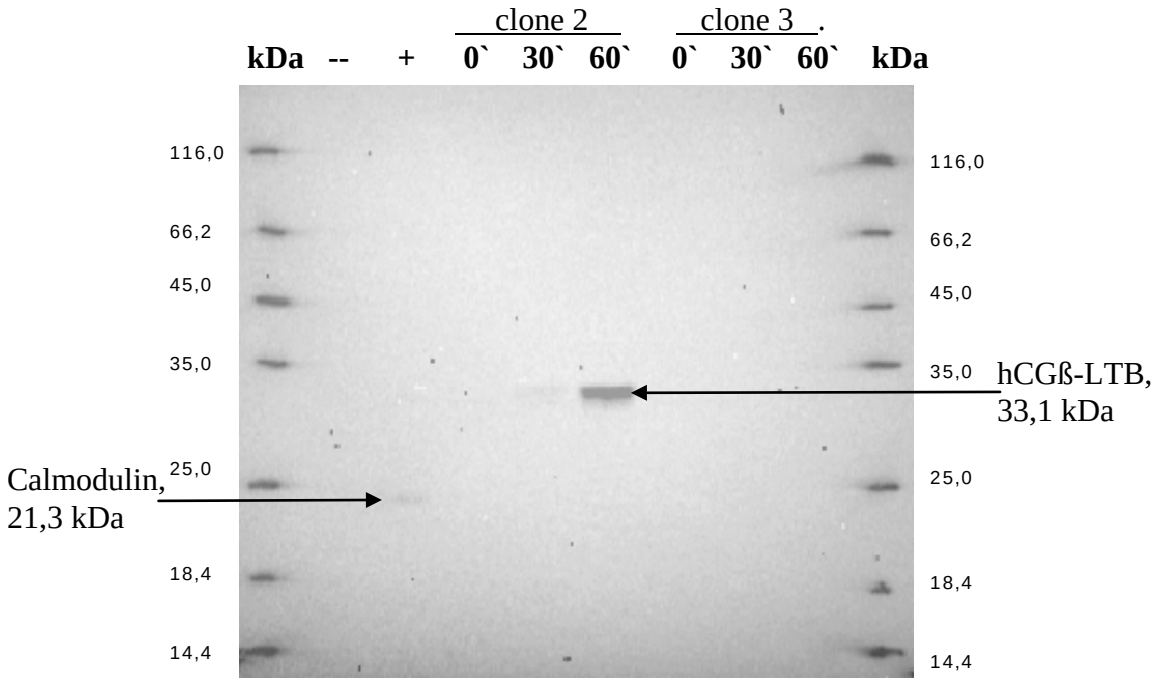

Fig. 19: Western blot analysis of hCGß-LTB expression from E. coli K12 C2988J (pBGK-CGL) clone 2 and 3.

-- E. coli K12 C2988J (pBGKA) 60 min; + E. coli K12 C2988J (pBAD/GIII/calmodulin) 60 min; Clone 2 is showing the best expression - a weak expresssion after 30 min and a stronger after 60 min of arabinose induction. At time point 0 min the hCGß-LTB expression was induced with L-arabinose.

The protein samples were analysed by western blot analysis. Expression of hCG-ß-LTB was detected using Anti-myc-HRP antibodies (see Fig. 19), detecting the myc fused to hCG-ß-LTB. Expression of hCG-ß-LTB was detected for clone 2. A weak expression was seen after 30 min, a clear band detected after 60 min of arabinose induction.
The expression of hCG-ß-LTB did not influence the bacterial growth negatively (data not shown).

4.1.2 Expression and lysis study

4.1.2.1 Expression and lysis study in *E. coli K12 NM522*

E. coli K12 NM522 was co-transformed with the expression plasmid pBGK-CGL and the lysis plasmid pGLysivb.
Positive clones were analysed by restriction analysis and used for the expression and lysis study.
Noseflasks with 25 ml LBv including gentamycin and kanamycin were used to test the positive clones 1-4 of *E. coli* K12 NM522 (pBGK-CGL, pGLysivb). The hCG-ß-LTB expression was induced with L-arabinose (final concentration of 0,2%) at OD_{600} ~ 0,2. The lysis induction was performed by temperature-upshift from 36°C to 42°C at OD_{600} ~ 0,5. During the experiment OD_{600} was measured, samples for cfu-determination and protein-samples for western blot analysis were taken.

As pictured below the bacterial growth was not affected by hCG-ß-LTB expresssion (see Fig. 20) and good lysis efficiency was reached (see Fig. 20).

The protein samples were analysed by western blotting. Expression of hCG-ß-LTB was detected by anti-myc-HRP antibodies. As shown in Fig. 21, there is hCG-ß-LTB antigen expression and the maximum lies at 20 min after induction of lysis. After lysis induction concentration of the protein was decreasing.

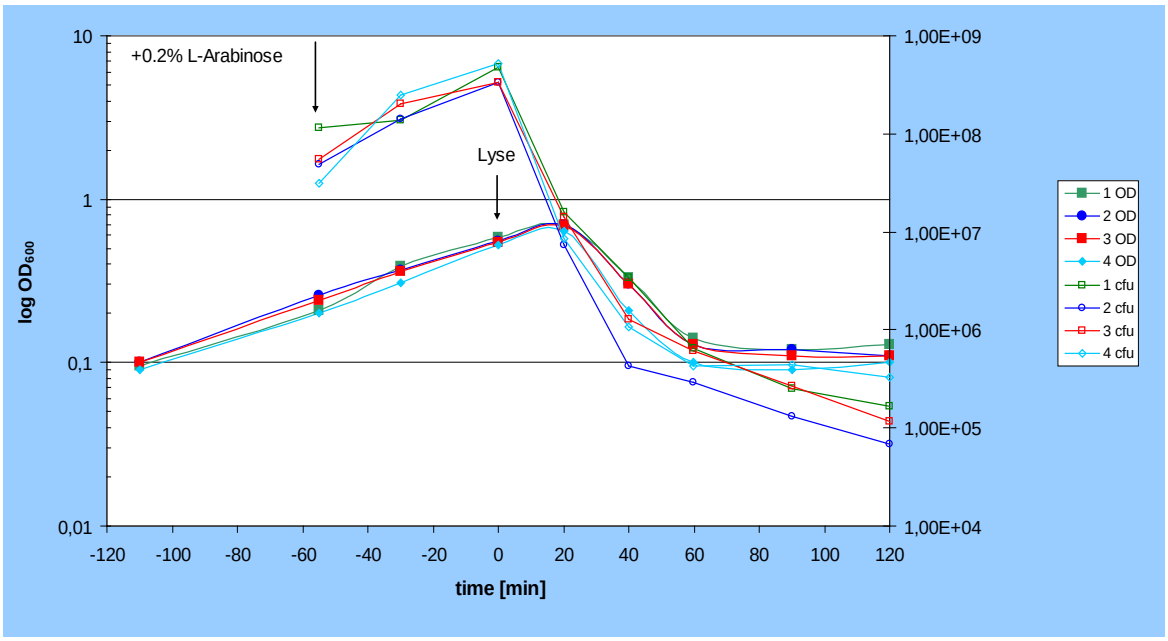

Fig. 20: Growth and lysis curve of E. coli* K12 NM522 *(pBGK-CGL, pGLysivb) clone 1 - 4.
Expression induction by addition of L-arabinose at OD_{600} ~ 0,2 (time point -55 min); Lysis induction – by temperature shift from 36°C to 42°C at OD_{600} ~ 0,5 (time point 0 min).

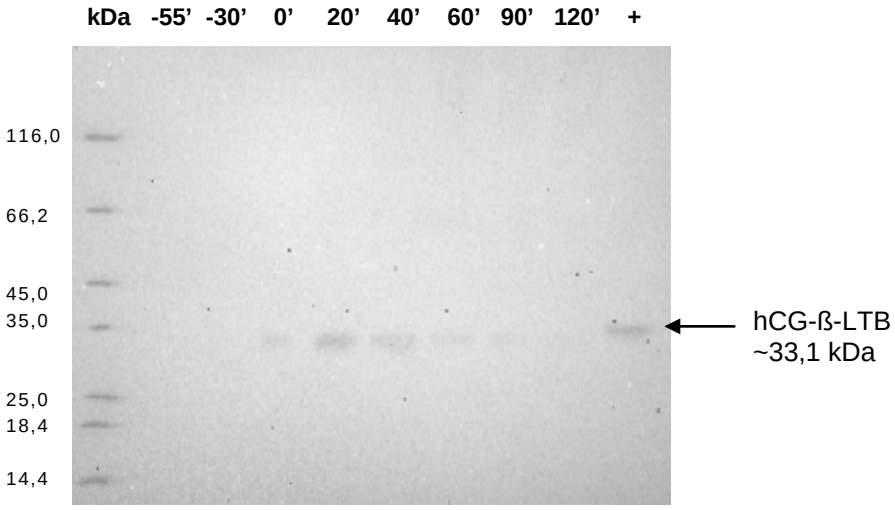

Fig. 21: Western blot analysis of hCG-ß-LTB fusion protein from E. coli* K12 NM522 *(pBGK-CGL, pGLysivb) clone 1.
The maximum of hCG-ß-LTB expression is 20 min after lysis induction, afterwards there is protein loss visible. Protein loss can have several reasons such as – uncorrect production of E-lysis tunnel or GIII transport to the PPS didn't work or degradation of hCG-ß-LTB by high amount of proteases within the E.coli strain. Protein hCG-ß-LTB induction by L-arabinose at time point - 55 min; lysis induction by temperature shift from 36°C to 42°C at time point 0 min; + positive control of a former hCG-ß-LTB expression.

The protein loss can have several reasons such as:

- Lysis process – correct production of E-lysis tunnel didn't work. One reason could be the nature of the bacterial strain or mechanical forces (physical conditions) which may have destroyed the cells.
- GIII-transport to the periplasmic space didn't work and cytoplasmic hCG-ß-LTB is lost during lysis process
- Degradation of hCG-ß-LTB by high amount of proteases within the *E. coli* strain.

The solution:

- New bacterial strain – *E. coli* K12 W3110 – protease deficient (lon^-)
- Production in fermenter – other conditions
- (New cloning strategy, with other vector and other transport system.)

4.1.2.2 Expression and lysis study in *E. coli* K12 W3110

E. coli K12 W3110 was co-transformed with the expression plasmid pBGK-CGL and the lysis plasmid pGLysivb.
Positive clones were analysed by restriction analysis and used for the expression and lysis study.
Noseflasks with 25 ml LBv complemented with gentamycin and kanamycin were used to test the positive clones of *E. coli* K12 W3110 (pBGK-CGL, pGLysivb). The hCG-ß-LTB expression was induced by addition of L-arabinose (final concentration of 0.2%) at OD_{600} ~ 0.2. The lysis induction was carried out by temperature upshift from 36 ° C to 42 ° C at OD_{600} ~ 0.5. During the experiment OD_{600} was measured, samples for cfu-determination and protein samples for western blot analysis were taken.
The bacterial growth was not negatively influenced by the L-arabinose induction and lysis curves showed good lysis efficiency (see Fig. 22).
Expression of hCG-ß-LTB-myc-His was detected by western blot analysis using anti-myc-HRP antibodies. Expression of hCG-ß-LTB is visible after addition of L-Arabinose and the protein concentration was not diminished after lysis induction (see Fig. 23).

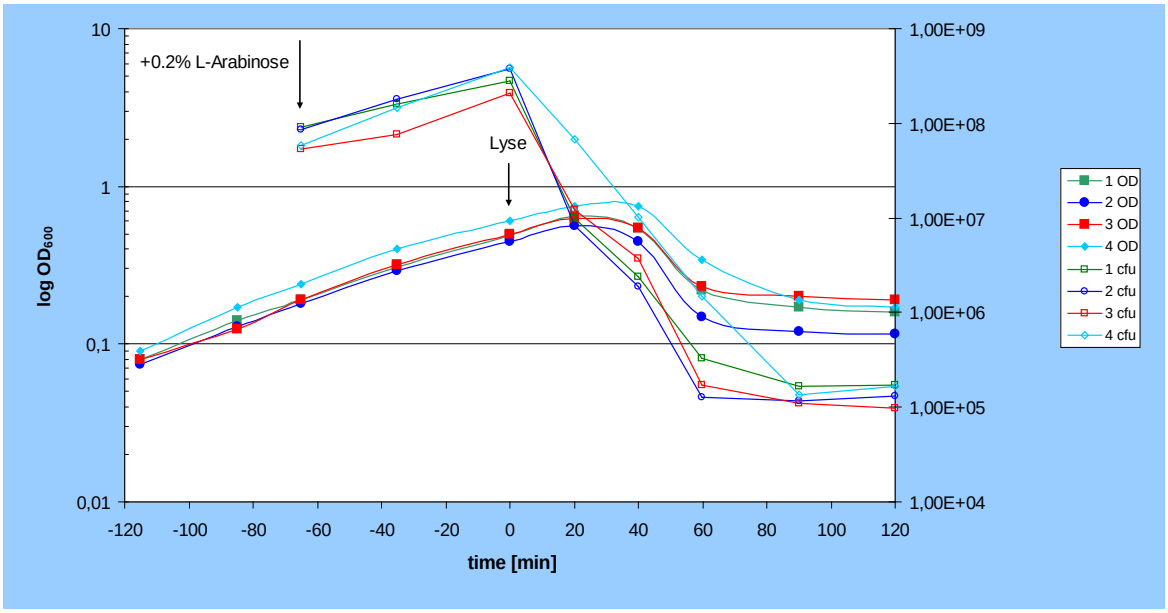

Fig. 22: Growth and lysis curves of E. coli* K12 W3110 *(pBGK-CGL, pGLysivb) clone 1-4.
Expression induction – by addition of L-arabinose at OD_{600} ~ 0,2; Lysis induction – by temperature upshift from 36°C to 42°C at OD_{600} ~ 0,5.

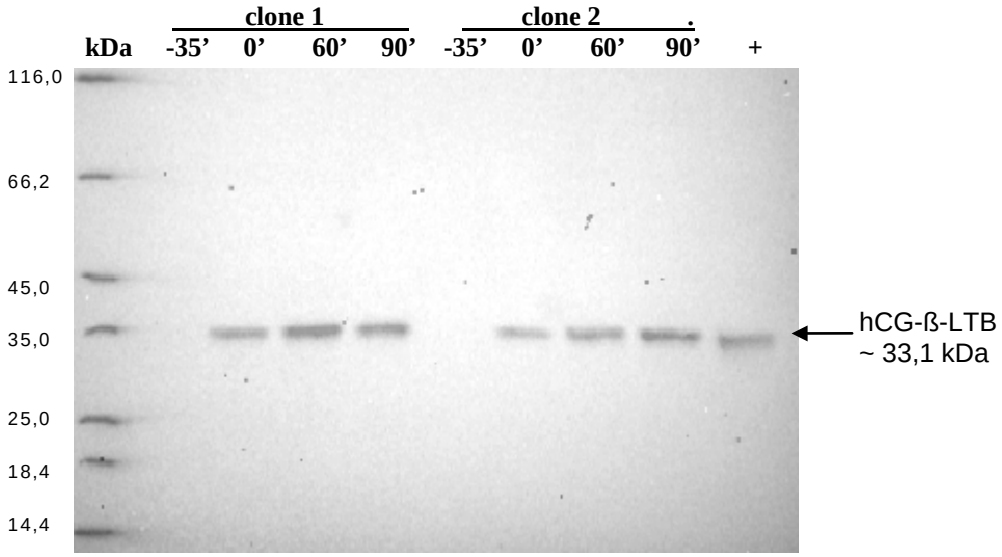

Fig. 23: Western blot analysis of hCG-ß-LTB expression from E. coli* K12 W3110 *(pBGK-CGL, pGLysivb) clone 1 and 2.
hCG-ß-LTB expression before (0 min) and after lysis (60 min; 90 min). After lysis induction there is no degradation of hCG-ß-LTB visible. Protein hCG-ß-LTB induction by L-arabinose at time point -60 min (not shown); Lysis induction by temperature upshift from 36°C to 42°C at time point 0 min; + positive control of a former hCG-ß-LTB expression.

4.1.3 Fermentation

Fermentation processes were performed as described in material and methods. For fermentation the following bacterial strains were used:

- *E. coli* K12 NM522 (pBGK-CGL, pGLysivb) - Clone 1
- *E. coli* K12 W3110 (pBGK-CGL, pGLysivb) - Clone 2

The fermentation approach was carried out in 22 liters LBv complemented with corresponding antibiotics - kanamycin (for the expression plasmid - pBGK-CGL) and gentamicin (for the lysis plasmid - pGLysivb). Protein expression was induced by addition of L-arabinose (final concentration of 0.2%). E-lysis induction was carried out by temperature shift from 35 ° C to 44 ° C 60 minutes after induction of protein expression. Killing of surviving bacteria was carried out 90 min after lysis induction by the addition of double concentration of tetracyclin and streptomycin (a standard method). The surviving bacteria can be killed also by a double dose of ß-Propiolacton (BPL). This new method of killing of remaining bacteria was in the test phase and therefore was not yet used. Harvesting and washing of BG were performed by tangential flow filtration (TFF). Lyophilized BG-material was controlled by sterility testings, western blotting analysis and quantification of the recombinant antigen.

4.1.3.1 Fermentation of *E. coli* K12 NM522 (pBGK-CGL, pGLysivb) – clone 1

Clone 1 of *E. coli* K12 NM522 (pBGK-CGL, pGLysivb), which has been tested before (see 4.1.2.1), was used for fermentation in 30 Liter fermenter.
The bacterial strain was inoculated to an OD_{600} ~ 0,4. At OD_{600} ~ 0,6 hCG-ß-LTB expression was induced by addition of L-arabinose (a final concentration of 0.2%) (see Fig. 24). The bacterial growth was not negatively affected by the hCG-ß-LTB expression. 60 minutes after L-arabinose addition E-lysis was induced by a temperature shift from 35 ° C to 44 ° C (see Fig. 24). The E-lysis took another 90 minutes and caused a cfu-drop from 10^8 to 10^4 (per ml). The surviving bacteria were killed by addition of double concentration of antibiotics (AB) for another 60 minutes (see Fig. 24).
OD and cfu values are shown in Fig. 24.
The lysis efficiency of this fermentation was 99,998%.

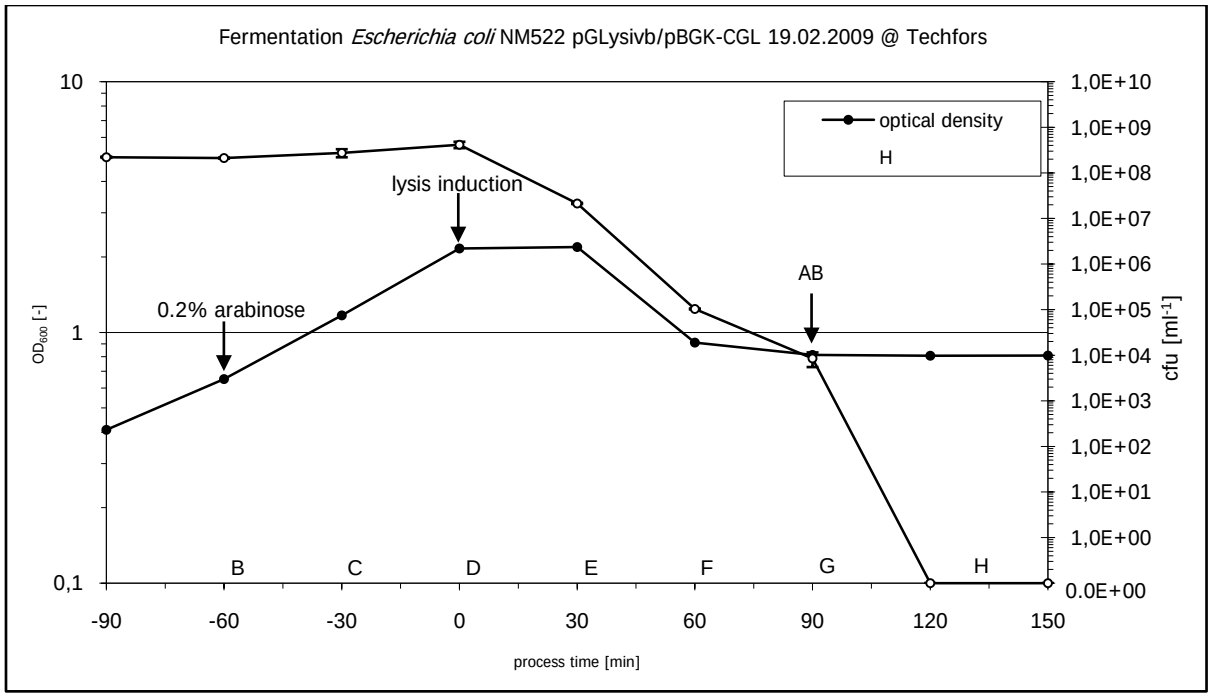

Fig. 24: Fermentation curve of E. coli* K12 NM522 *(pBGK-CGL, pGLysivb) – clone 1.
hCG-ß-LTB protein induction by 0,2% L-arabinose at OD600 ~ 0,6; Lysis induction by temperature shift from 35°C to 44°C; Killing by streptamycin and tetracyclin double concentration (AB).
Abbreviations: -●- optical density; -○- cfu.

The IRIS software documents all important parameters during the fermentation such as stirrer, pH, oxygen, temperature and flow. The IRIS curve showing all documented parameters can be seen in Fig. 25.

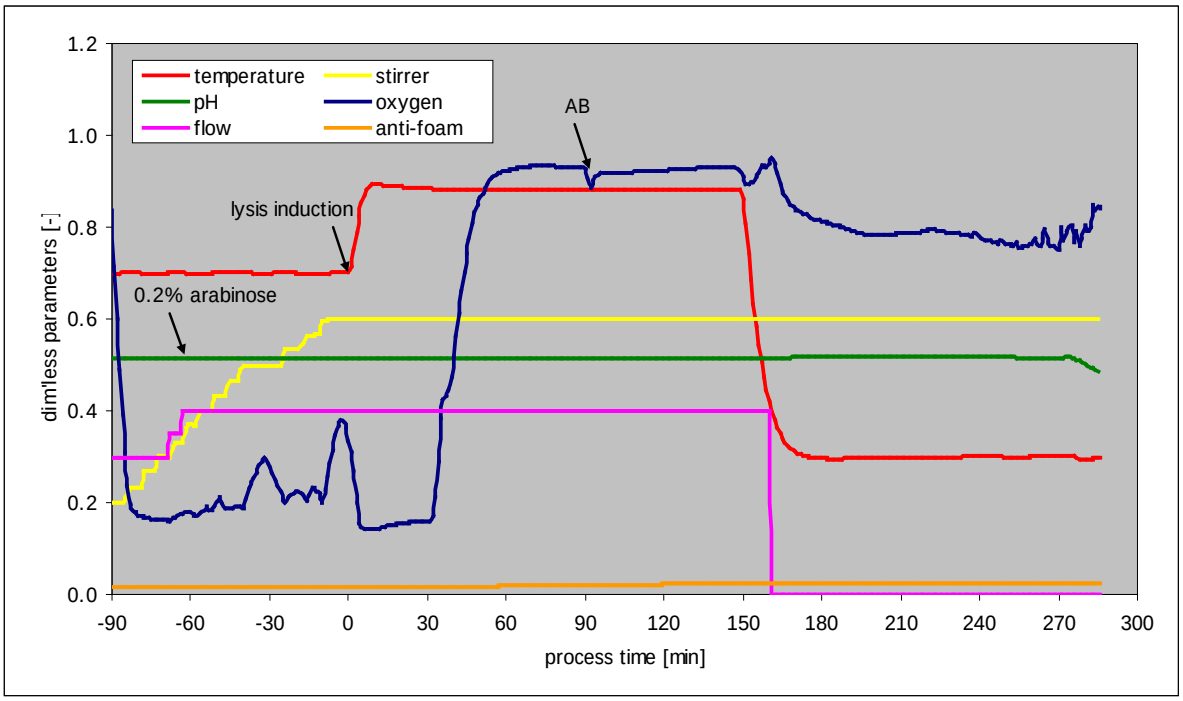

Fig. 25: IRIS curve of the fermentation of E. coli* K12 NM522 *(pBGK-CGL, pGLysivb) – clone 1

Main data of the fermentation process can be found also in the fermentation data sheet (Fig. 26):

Pre-culture	
Volume: **4*500 ml**	Additives: **Gent / Kan**
Medium type: **LBv**	Other: -
Date: **2009/02/19**	Clone: **c1** (12.12.2009, WS: 06.02.2009 by IHO)
Starting time: **08:20**	Strain: ***Escherichia coli* NM522**
End time: **12:20**	Plasmids: **pGLysivb/pBGK-CGL**
ON culture OD: **2.251 / 0.541**	Recombinant Protein Expression: **HCGß-LTB**
Inoc. Volume: **1.60 l**	Expression Induction: **0.2% arabinose**
Medium: **LBv**	Expression Induction Timepoint: **B**
Antibiotics: **Gent/Kan**	Lysis Induction: **44°C**
Temperature: **35°C**	Lysis Induction Timepoint: **D**
Total Volume: **~ 22 L**	Killing**: Strep, Tet double concentration (G)**
Acid: F.A.: **33 ml**	Volume harvested: **20 l**
Base: A.W.: **66 ml**	Harvested by: **TFF** (both steps + washing)
Antifoam A: **25 ml**	OD seperator flow: **< 0.000**
E-Blot: -	Yield: **7890 mg**
R-Blot: **OK (by IHO)**	Particles -/ mg: **1.05×10^9**
RT: -	Sterility: **OK (by AFA)**
Microscopy: **okay**	Efficiency: **99.998 %**

Fig. 26: Fermentation data sheet of the fermentation of E. coli* K12 NM522 *(pBGK-CGL, pGLysivb) – clone 1

The bacterial ghosts were harvested by tangential flow filtration (TFF) and dried by lyophylisation. The fermentation yield was 7890 mg lyophilized material with calculated 1.05 x 10^9 particles per mg (see Fig. 26).

Expression of hCG-ß-LTB-myc-His was detected by western blot analysis using anti-His-HRP antibodies. Expression of hCG-ß-LTB (33,1 kDa) was seen after addition of L-arabinose. The concentration of the protein was not diminished after lysis induction (see Fig. 27) which is a proof of an intact periplasmic transport of hCG-ß-LTB.

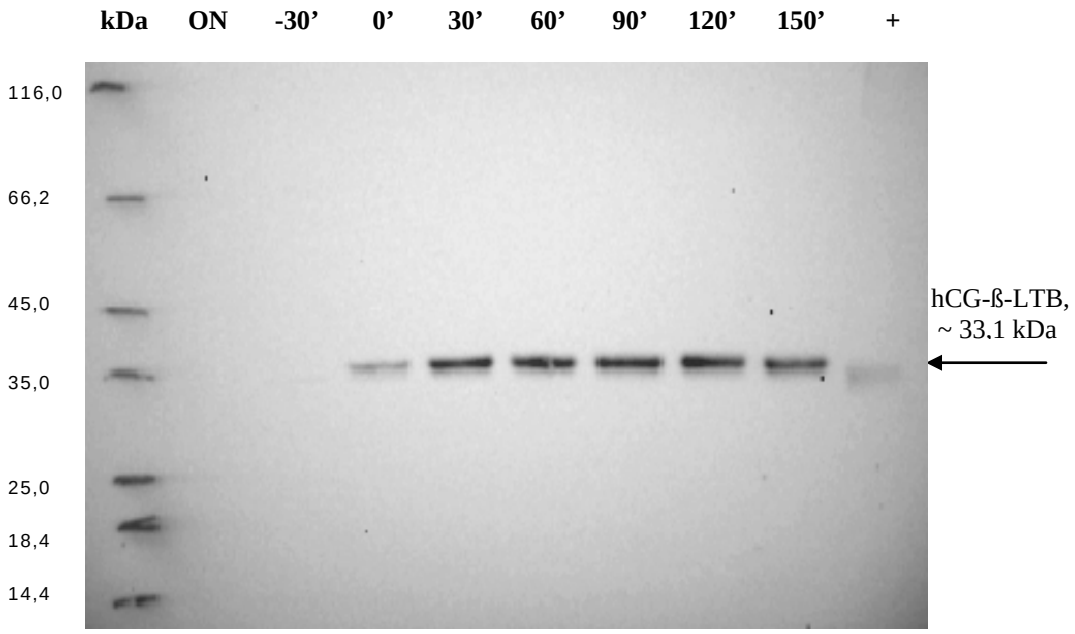

Fig. 27: Western blot analysis of hCG-ß-LTB expression from fermentation of E. coli* K12 NM522 *(pBGK-CGL, pGLysivb) clone 1. *hCG-ß-LTB protein is detectable 60 min after protein induction using anti-His-HRP antibodies and still present 150 min after lysis induction. Protein induction by L-arabinose at time point -60 min (not shown); Lysis induction by temperature shift from 35°C to 44°C at time point 0 min; + positive control of a former hCG-ß-LTB expression. ON – over night culture E. coli* K12 NM522 *(pBGK-CGL, pGLysivb) clone 1*

4.1.3.2 Fermentation of *E. coli* K12 W3110 (pBGK-CGL, pGLysivb) – clone 2

Clone 2 of *E. coli* K12 W3110 (pBGK-CGL, pGLysivb), which has been tested before (see 4.1.2.2), was used for fermentation in 30 liter fermenter.

The expression of hCG-ß-LTB protein was induced by addition of L-arabinose (final concentration of 0.2%) at OD_{600} ~ 0.6 (see Fig. 28). The bacterial growth was not negatively affected by the hCG-ß-LTB expression. 60 minutes after L-arabinose addition E- lysis was induced by a temperature shift from 35 ° C to 44 ° C (see Fig. 28). The E-lysis took another 90 minutes and caused a cfu-drop from 10^8 to 10^4 (per ml). The surviving bacteria were killed again by addition of double concentration of antibiotics (AB) for another 60 minutes (see Fig. 28). OD and cfu values are shown in Fig. 28.

The lysis efficiency of this fermentation was 99.995%.

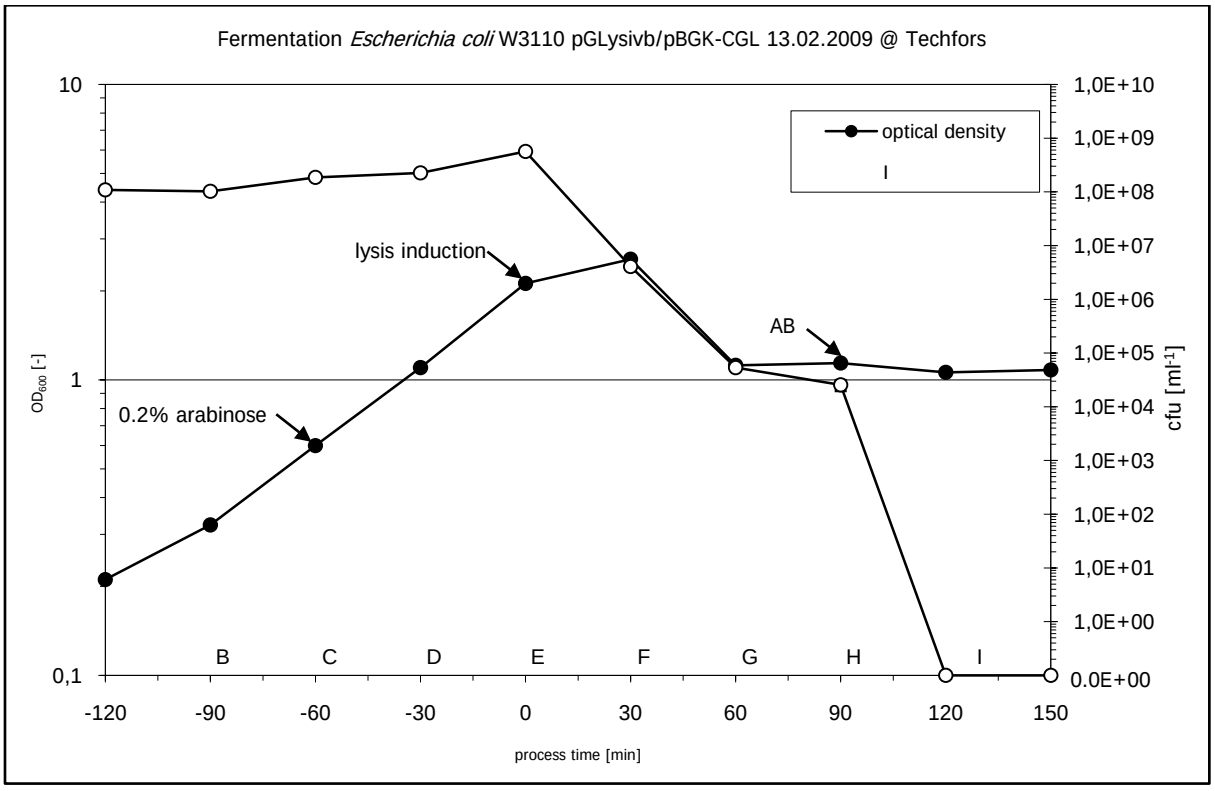

Fig. 28: Fermentation curves of E. coli* K12 W3110 *(pBGK-CGL, pGLysivb) – clone 2
hCG-ß-LTB protein induction by 0,2% L-arabinose at OD_{600} ~ 0,6; Lysis induction by temperature shift from 35°C to 44°C; Killing by Streptomycin and Tetracyclin double concentration (AB).
Abbreviations: -●- optical density; -○- cfu.

The IRIS curve showing all important fermentation parameters is shown in Fig. 29.

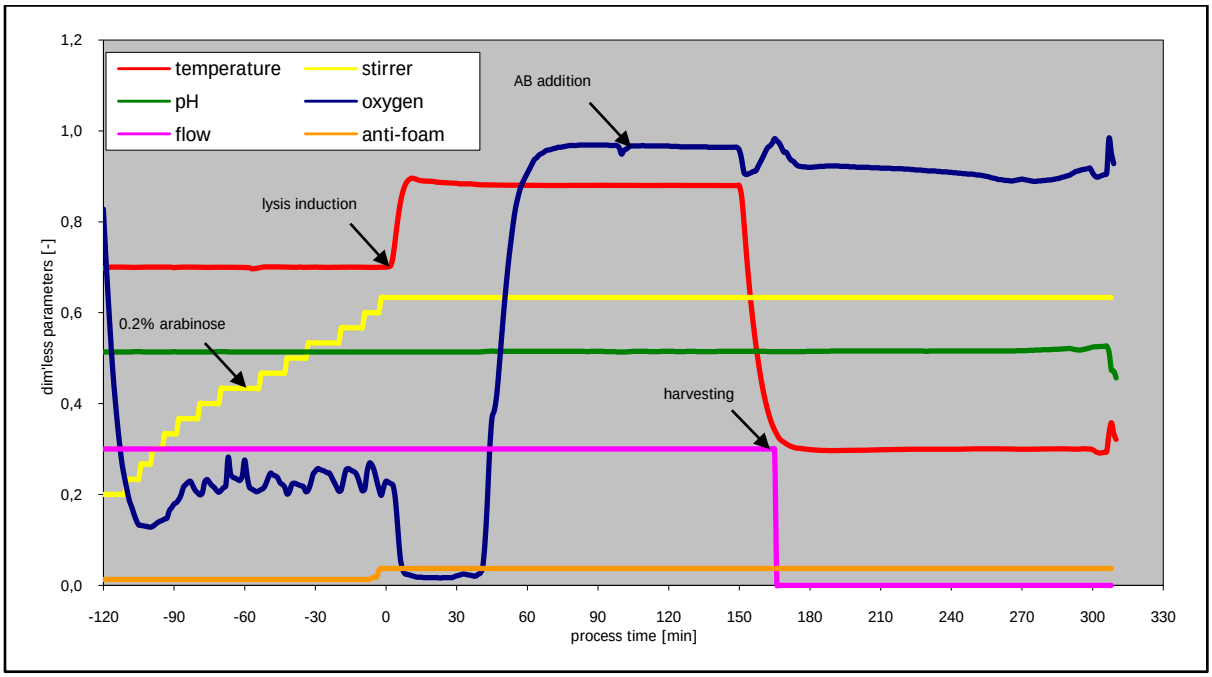

Fig. 29: IRIS curve of the fermentation of E. coli* K12 W3110 *(pBGK-CGL, pGLysivb) – clone 2

Main data of the fermentation process can be found also in the fermentation data sheet (Fig. 30):

The bacterial ghosts were harvested by tangential flow filtration (TFF) and dried by lyophilisation. The fermentation yield was 9453 mg of lyophilized material with calculated 1.19 x 10^9 particles per mg (see Fig. 30).

Expression of hCG-ß-LTB was detected by western blot analysis using anti-myc-HRP antibodies. Expression of hCG-ß-LTB (33,1 kDa) was seen after addition of L-arabinose (see Fig. 31). The concentration of the protein was not decreasing after lysis induction which is a proof of an intact periplasmic transport of hCG-ß-LTB.

Pre-culture	
Volume: **4*500 ml**	Additives: **Gent / Kan**
Medium type: **LBv**	Other: -
Date: **2009/02/13**	Clone: **c2** (28.01.2009, WS: 05.02.2009 by IHO)
Starting time: **08:30**	Strain: ***Escherichia coli* W3110**
End time: **13:00**	Plasmids: **pGLysivb/pBGK-CGL**
ON culture OD: **2.280 / 0.561**	Recombinant Protein Expression : **HCGß-LTB**
Inoc. Volume: **0.78 l**	Expression Induction: **0.2% arabinose**
Medium: **LBv**	Expression Induction Timepoint: **C**
Antibiotics: **Gent, Kan**	Lysis Induction: **44°C**
Temperature: **35°C**	Lysis Induction Timepoint: **E**
Total Volume: **~ 22 L**	Killing**: Strep, Tet double concentration (H)**
Acid: F.A.: **30.3 ml**	Volume harvested: **20 l**
Base: A.W.: **62.6 ml**	Harvested by: **TFF** (both steps + washing)
Antifoam A: **36.8 ml**	OD seperator flow: **< 0.000**
E-Blot: **OK (by IHO)**	Yield: **9453 mg**
R-Blot: **OK (by IHO)**	Particles -/ mg: **1.19×10^9**
RT: -	Sterility: **OK (by AFA)**
Microscopy: **okay**	Efficiency: **99.995 %**

Fig. 30: Fermentation data sheet of the fermentation of E. coli* K12 W3110 *(pBGK-CGL, pGLysivb) – clone 2

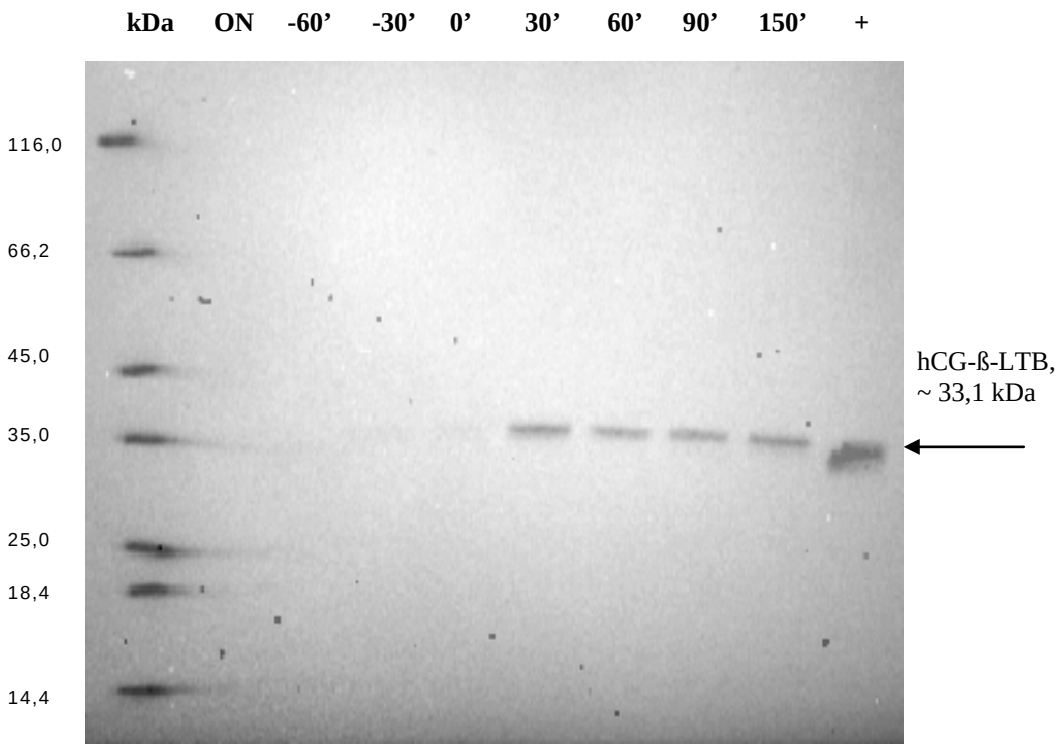

Fig. 31: Western blot analysis of hCG-ß-LTB expression from fermentation of E. coli* K12 W3110 *(pBGK-CGL, pGLysivb) clone 2. *hCG-ß-LTB protein is detectable 30 min after protein induction using anti-His-HRP antibodies and still present 150 min after lysis induction. Protein induction by L-arabinose at -60 min; Lysis induction by temperature shift from 35°C to 44°C at 0 min; + positive control of a former hCG-ß-LTB expression; ON – over night culture of E. coli* K12 W3110 *(pBGK-CGL, pGLysivb) clone 2.*

4.1.3.3 Quantification of recombinant hCG-ß-LTB from *E. coli* Ghosts produced by fermentation

Recombinant protein hCG-ß-LTB of lyophilized bacterial ghosts was quantified using western blot analysis and the Quantity One program from BioRad (Hercules, CA, USA). The positope (Invitrogen) was used as standard for quantification.
The positope with a molecular weight of 53 kDa is delivered with a concentration of a 25 ng/µl. A serial dilution (1:2) of the positope was made and was loaded according to the following amounts for the standard curve calculation:

- 500 ng Standard 1
- 250 ng Standard 2
- 125 ng Standard 3
- 62,5 ng Standard 4

The lyophilized hCG-ß-LTB bacterial ghosts were diluted as described in materials and methods and loaded on the gel:

- 5 µg of BG's *E. coli* K12 NM522 or *E. coli* K12 W3110
- 10 µg of BG's *E. coli* K12 NM522 or *E. coli* K12 W3110
- 15 µg of BG's *E. coli* K12 NM522 or *E. coli* K12 W3110

For the membrane development anti-myc-HRP antibodies were used (see Fig. 32). The quantification of the hCG-ß-LTB protein was performed using the QuantityOne Software of the ChemiDocXRS program.

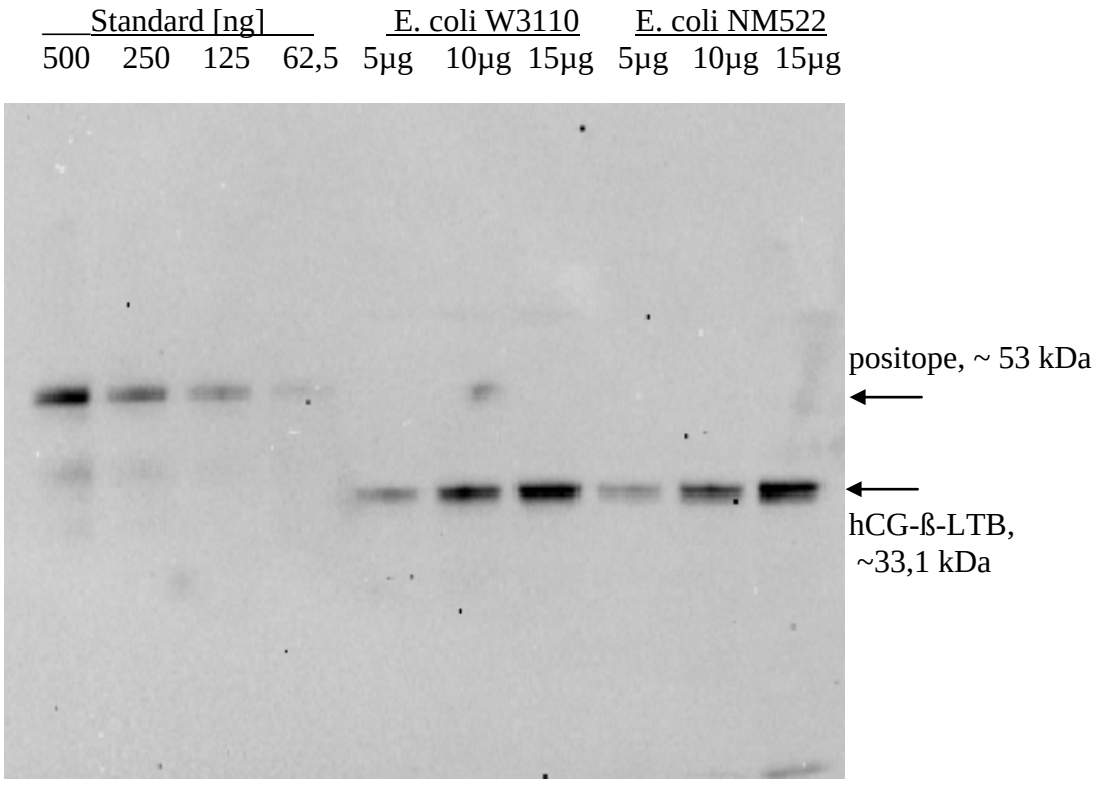

Fig. 32: Quantification western blot analysis of lyophilised hCG-ß-LTB bacterial ghosts from fermentation of E. coli* K12 NM522 *(pBGK-CGL, pGLysivb) clone 1 and E. coli* K12 W3110 *(pBGK-CGL, pGLysivb) clone 2. *As standard – positope (Invitrogen).*

The quantificaton results are shown on the quantification curve in Fig. 33.
Based on the standard curve the concentration of hCG-ß-LTB protein in BG of *E. coli* K12 NM522 and in BG of *E. coli* K12 W3110 was calculated:

- 5 µg of BG's *E. coli* K12 NM522 38,49 ng hCG-ß-LTB / µg BG
- 10 µg of BG's *E. coli* K12 NM522 40,52 ng hCG-ß-LTB / µg BG
- 15 µg of BG's *E. coli* K12 NM522 54,51 ng hCG-ß-LTB / µg BG

The resulting average was 44,51 ng hCG-ß-LTB / µg BG of *E. coli* K12 NM522.

- 5 µg of BG's *E. coli* K12 W3110 40,27 ng hCGßLTB / µg BG
- 10 µg of BG's *E. coli* K12 W3110 53,47 ng hCGßLTB / µg BG
- 15 µg of BG's *E. coli* K12 W3110 55,52 ng hCGßLTB / µg BG

The resulting average was 49,75 ng hCGßLTB / µg BG of *E. coli* K12 W3110.

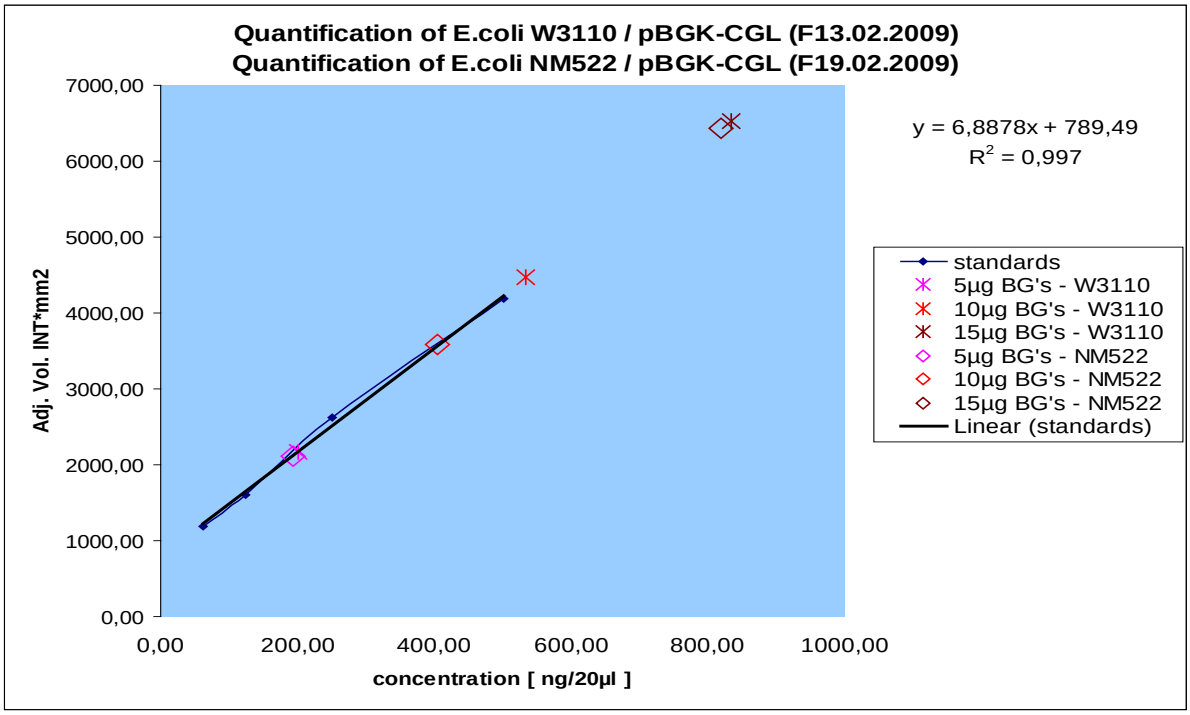

Fig. 33: Quantification curve of hCG-ß-LTB expressing BG's of fermentations

- E. coli K12 NM522 *(pBGK-CGL, pGLysivb) clone 1 with resulting average of 44,51 ng hCG-ß-LTB / µg BG*

- E. coli K12 W3110 *(pBGK-CGL, pGLysivb) clone 2 with resulting average of 49,75 ng hCG-ß-LTB / µg BG.*

The lyophilized hCG-ß-LTB bacterial ghosts of *E. coli* K12 NM522 were sended to Dr. Talwar (Talwar Research Foundation, New Delhi, India) for animal trials.

4.2 Lysis plasmids

4.2.1 Lysis plasmid – pGLysivb-2x

Lysis plasmid pGLysivb-2x is a derivate of pGLysivb carrying a second temperature inducibel lysis cassette. The idea behind the construction of this plasmid was that two instead of one lysis cassettes should due to higher lysis efficiency as well to a more rapid lysis induction.

4.2.1.1 Construction of lysis plasmid pGLysivb-2x

The lysis cassette was amplified by PCR from pGLysivb, incorporating restriction sites for NcoI and XhoI. After cutting with these two enzymes the fragment (1380 bp) was ligated to the pGLysivb fragment (5788 bp) cutted with NcoI and XhoI (see Fig. 34).

For the PCR following primers were used:

Eivb(NcoI)fwd: 5` AAT CCA TGG TCA GCC AAA CGT CTC TTC 3´
Tm=54°C
Eivb(XhoI)rev: 5` AAT CTC GAG TCA TTC GTG CCA TTC GAT T 3´
Tm=56°C
…. Restriction sequence
___ Binding region within the insert fragment

PCR was carried out according to standard protocol using:

- Pfu - Polymerase (2,5 u/µl) from Fermentas
- 10x Pfu - buffer (+$MgCl_2$) system

After transformation of the ligation mix into *E. coli* K12 C2988J, positive clones were analysed by restriction digests and stored as glycerin stocks.

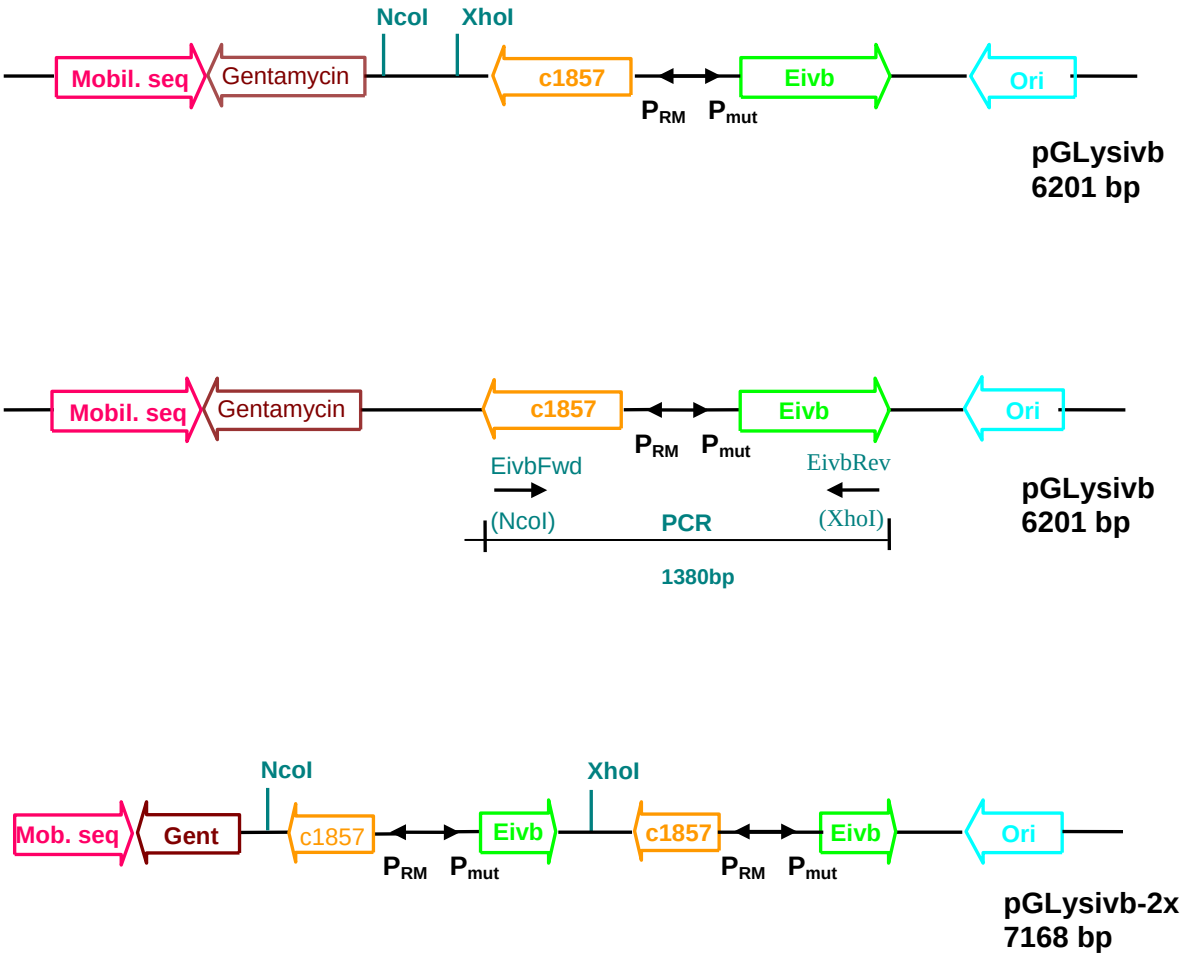

Fig. 34: Cloning strategy of pGLysivb-2x. *pGLysivb is the backbone plasmid and donor plasmid for the lysis cassette. pGLysivb-2x contains two independent lysis cassettes which are under control of the λpR-cI857, each.*

4.2.1.2 Lysis control study of *E. coli* K12 C2988J (pGLysivb-2x)

Experiments are carried out in the nose flasks with 25 ml of LBv and the corresponding antibiotics as described in material and methods. Two positive clones of *E. coli* K12 C2988J (pGLysivb-2x) were compared with *E. coli* K12 C2988J (pGLysivb) control in their lysis behaviour. Lysis induction was performed by temperature shift from 36°C to 42°C at OD_{600} ~ 0,3 - 0,4. Uninduced samples were included as negativ controls.

Figure 35 shows the growth and lysis curves of two cultures of *E. coli* K12 C2988J (pGLysivb-2x) and of the positive control *E. coli* K12 C2988J (pGLysivb).

E. coli K12 C2988J (pGLysivb-2x) clones showed a lower lysis efficiency compared to the positive control clone *E. coli* K12 C2988J (pGLysivb) (see Fig. 35 and Tab. 3).

Lysis efficiency is about 1 log lower than for the *E. coli* K12 C2988J (pGLysivb) control (see Tab. 3).

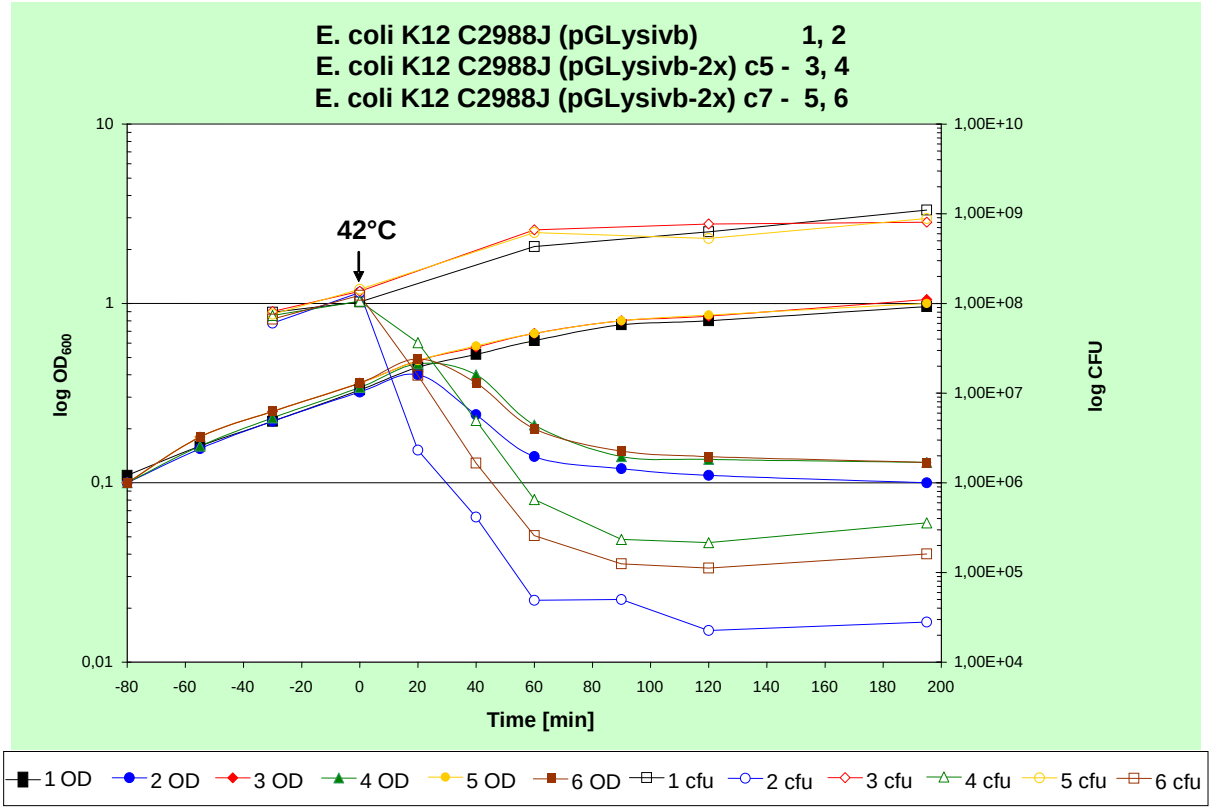

Fig. 35: Growth (OD) and lysis (cfu) curves of E. coli* K12 C2988J *(pGLysivb) (1, 2); E. coli* K12 C2988J *(pGLysivb-2x) clone 5 (3, 4); E. coli* K12 C2988J *(pGLysivb-2x) clone 7 (5, 6). *The arrow indicates the time point of lysis induction by temperature shift from 36°C to 42°C for samples 2, 4, 6. Uninduced controls 1, 3, 5. E. coli K12 C2988J (pGLysivb-2x) clones show a lower lysis efficiency.*

cfu	Highest cfu	Lowest cfu	Lysis efficiency
E. coli K12 C2988J (pGLysivb) – positive control	1,32 x 10^8	2,25 x 10^4	99,98%
E. coli K12 C2988J (pGLysivb-2x) clone 5	1,06 x 10^8	2,15 x 10^5	99,79%
E. coli K12 C2988J (pGLysivb-2x) clone 7	1,23 x 10^8	1,12 x 10^5	99,90%

Tab. 3: Lysis efficiency of E. coli* K12 C2988J *(pGLysivb) and E. coli* K12 C2988J *(pGLysivb-2x)

4.2.2 Lysis plasmid – pGLMivb

Lysis plasmid pGLMivb is a modification of pGLysivb, where the temperature inducible λpR-cI857 promotor repressor system was exchanged by a chemical inducible LacIq-Repressor-Ptac-Promotor system (lysis-induction with IPTG). pGLMivb was designed for bacteria with a growth optimum above 36°C.

4.2.2.1 Construction of lysis plasmid pGLMivb

The LacIq-Repressor-Ptac-Promotor cassette was amplified by PCR from pMal-p2x incorporating restriction sites for XhoI and SbfI. After cutting with these two enzymes the fragment (1487 bp) was ligated to the pGLysivb fragment (5193 bp) cutted with XhoI and SbfI (see Fig. 36).

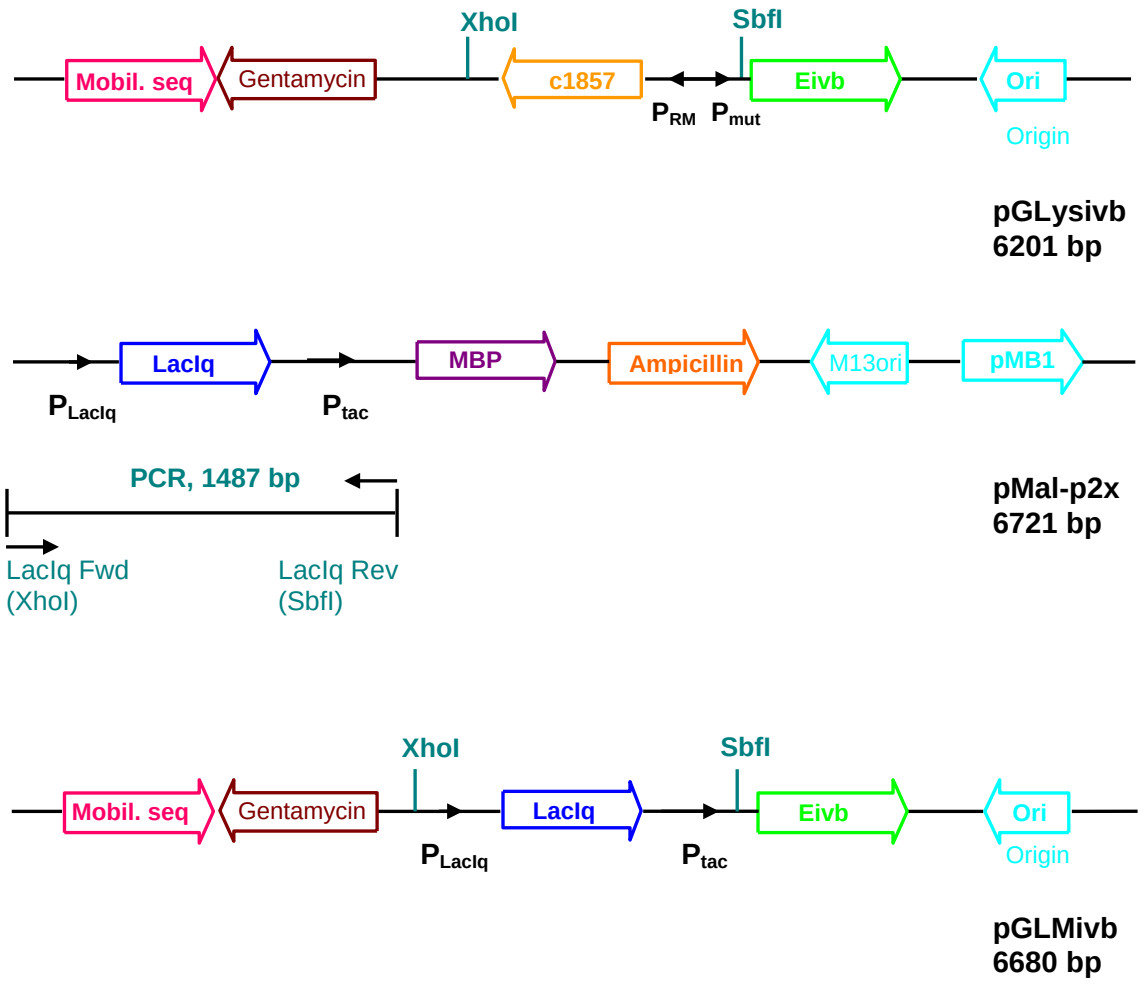

Fig. 36: Cloning strategy of lysis plasmid pGLMivb. *pGLysivb is the backbone plasmid. pMal-p2x is the donor plasmid for the LacIq-repressor-Ptac-promoter cassette. pGLMivb contains the lysis gene Eivb under control of the LacIq-repressor-Ptac-promoter system.*

For the PCR following primers were used:

LacIq-XhoI-fwd: 5` ATA CTC GAG CAC CAT CGA ATG GTG CAA A 3´
Tm=56°C

LacIq-SbfI-rev: 5` ATA CCT GCA GGA CTG GCT GTT TCC TGT 3´
Tm=56°C

…. Restriction sequence
___ Binding region within the insert fragment

PCR was carried out according to standard protocol using:

- Pfu - Polymerase (2,5 u/µl) from Fermentas
- 10x Pfu - buffer (+$MgCl_2$) system

After transformation of the ligation mix into *E. coli* K12 C2988J, positive clones were analysed by restriction digests and stored as glycerin stocks.

4.2.2.2 Lysis control study of E. coli K12 C2988J (pGLMivb)

Experiments are carried out in the nose flasks with 25 ml of LBv and the corresponding antibiotics as described in material and methods. One positive clone of *E. coli* K12 C2988J (pGLMivb) was compared with *E. coli* K12 C2988J (pBBR1MCS5) control in lysis behaviour. Plasmid pBBR1MCS5 (backbone of pGLysivb) does not carry the gene *Eivb* and therefore it does not undergo E-lysis. Lysis induction was performed by addition of IPTG (final concentration of 5mM) at OD_{600} ~ 0,3 - 0,4. Uninduced samples were included as negative controls.

Fig. 37 shows the growth and lysis curves of *E. coli* K12 C2988J (pGLMivb) and the negative control *E. coli* K12 C2988J (pBBR1MCS5). The lysis of *E. coli* K12 C2988J (pGLMivb) is visible immediately after IPTG addition by decrease in cfu but the lysis efficiency is lower in contrast to *E. coli* K12 C2988J (pGLysivb) (see Tab. 4). One disadvantage is a rapid regeneration of non-lysed cells after endpoint of lysis.

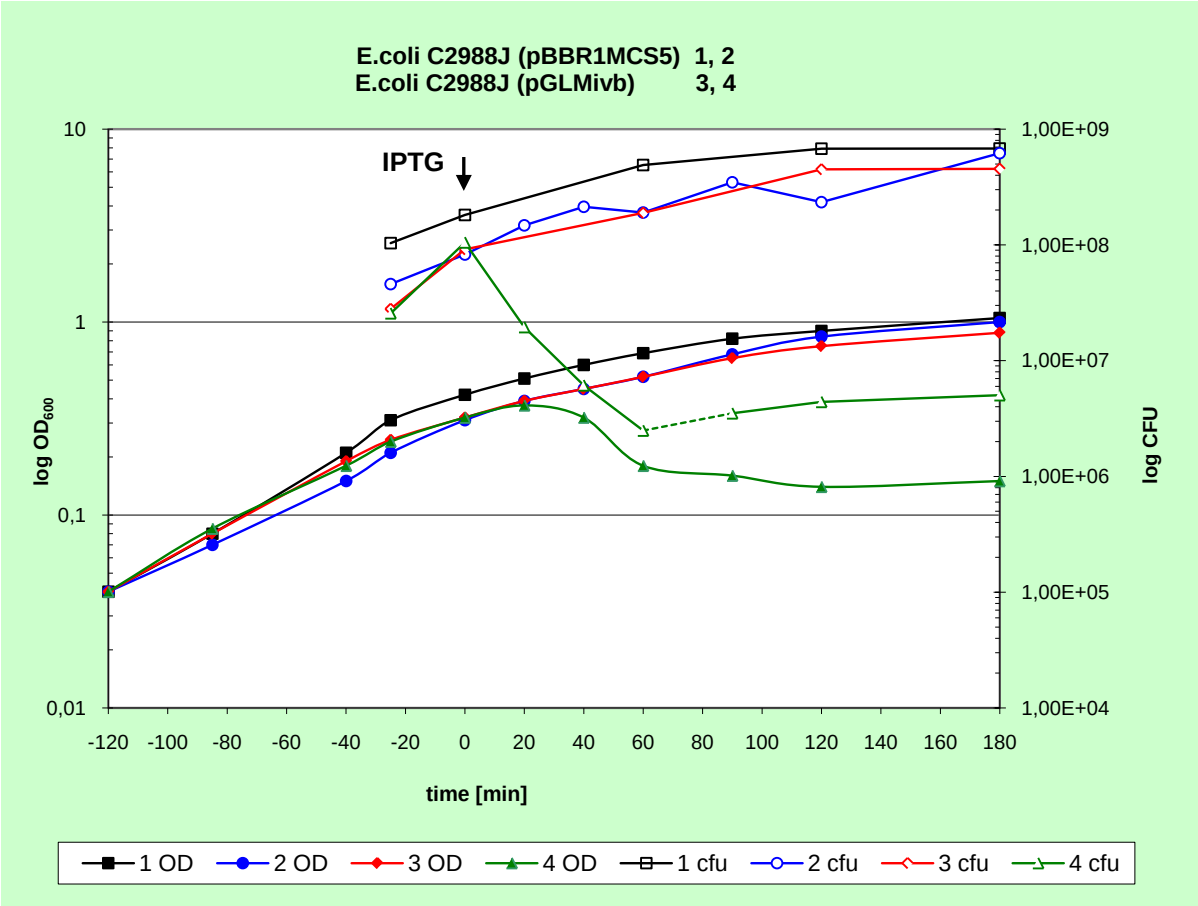

Fig. 37: Growth (OD) and lysis (cfu) curves of E. coli* K12 C2988J *(pBBR1MCS5) (1, 2); E. coli* K12 C2988J *(pGLMivb) (3, 4). *The arrow indicates the time point of lysis induction by IPTG addition (final concentration 5mM) for samples 2, 4. Uninduced controls – 1, 3. The lysis after IPTG addition is visible only by E. coli K12 C2988J (pGLMivb) – sample 4.*

cfu	Highest cfu	Lowest cfu	Lysis efficiency
E. coli K12 C2988J (pGLysivb)	1,32 x 10^8	2,25 x 10^4	99,98%
E. coli K12 C2988J (pGLMivb)	1,05 x 10^8	2,49 x 10^6	97,62%

Tab. 4: Lysis efficiency of E. coli* K12 C2988J *(pGLysivb) and E. coli* K12 C2988J *(pGLMivb)

A western blot was performed to detect the protein Eivb expression using Strep-HRP. Fig. 38 shows the expression of Eivb protein and cytoplasmic BCCP. Only the IPTG-induced *E. coli* K12 C2988J (pGLMivb) showed E-lysis which is also visible in Eivb exprcession after lysis induction. No Eivb expression was detected for the control *E. coli* K12 C2988J (pBBR1MCS5).

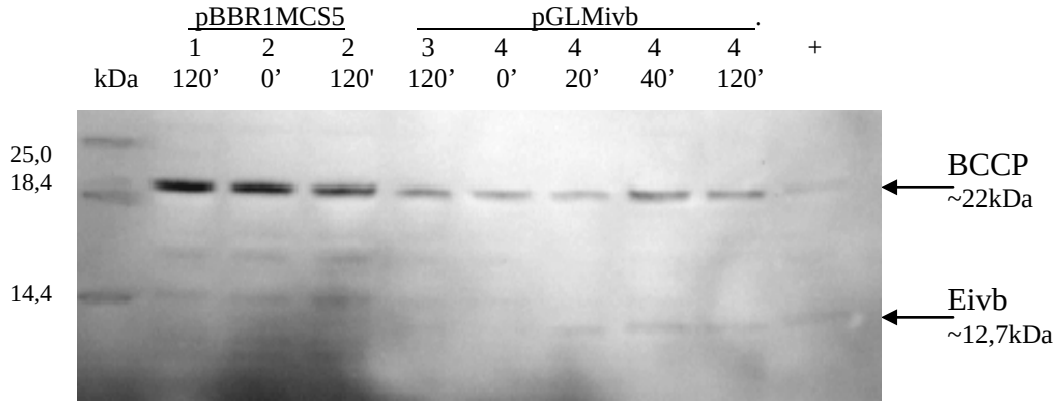

Fig. 38: Western blot showing Eivb protein and cytoplasmic BCCP from lysis control study of E. coli* K12 C2988J *(pGLMivb). *1 – E. coli* K12 C2988J *(pBBR1MCS5) without IPTG addition; 2 - E. coli* K12 C2988J *(pBBR1MCS5) with addition of IPTG at timepoint 0 min; 3 - E. coli* K12 C2988J *(pGLMivb) without lysis induction; 4 - E. coli* K12 C2988J *(pGLMivb) with lysis induction by IPTG at timepoint 0 min – 20 min after lysis induction Eivb protein expression is visible; + positive control of a former Eivb expression.*

4.2.2.3 Lysis consistency study of *E. coli* K12 NM522 (pGLMivb)

Bacterial strain *E. coli* K12 NM522 was used for the BG production.Therefore the lysis behavior of pGLMivb in *E. coli* K12 NM522 was studied in a consistency study. 9 different clones of *E. coli* K12 NM522 (pGLMivb) were compared. The clone with the best lysis profil was selected for BG production by fermentation. Experiments are carried out in the nose flasks with 25 ml of LBv and the corresponding antibiotics as described in material and methods.

E. coli K12 NM522 (pGLMivb) clone 8 showed the best lysis profil as shown in Fig. 39 and Tab. 5. Immediately after IPTG addition a decrease in cfu occurred. Lysis end was reached already 40 min after IPTG addition. Further incubation resulted in regeneration of not lysed cells.

A western blot was performed for qualitative analysis of Eivb protein and cytoplasmic BCCP. As shown in Fig. 40, all 9 clones of *E. coli* K12 NM522 (pGLMivb) show about the same expression level of Eivb protein. Concentration of cytoplasmic BCCP is very weakly as it is expelled during the lysis process.

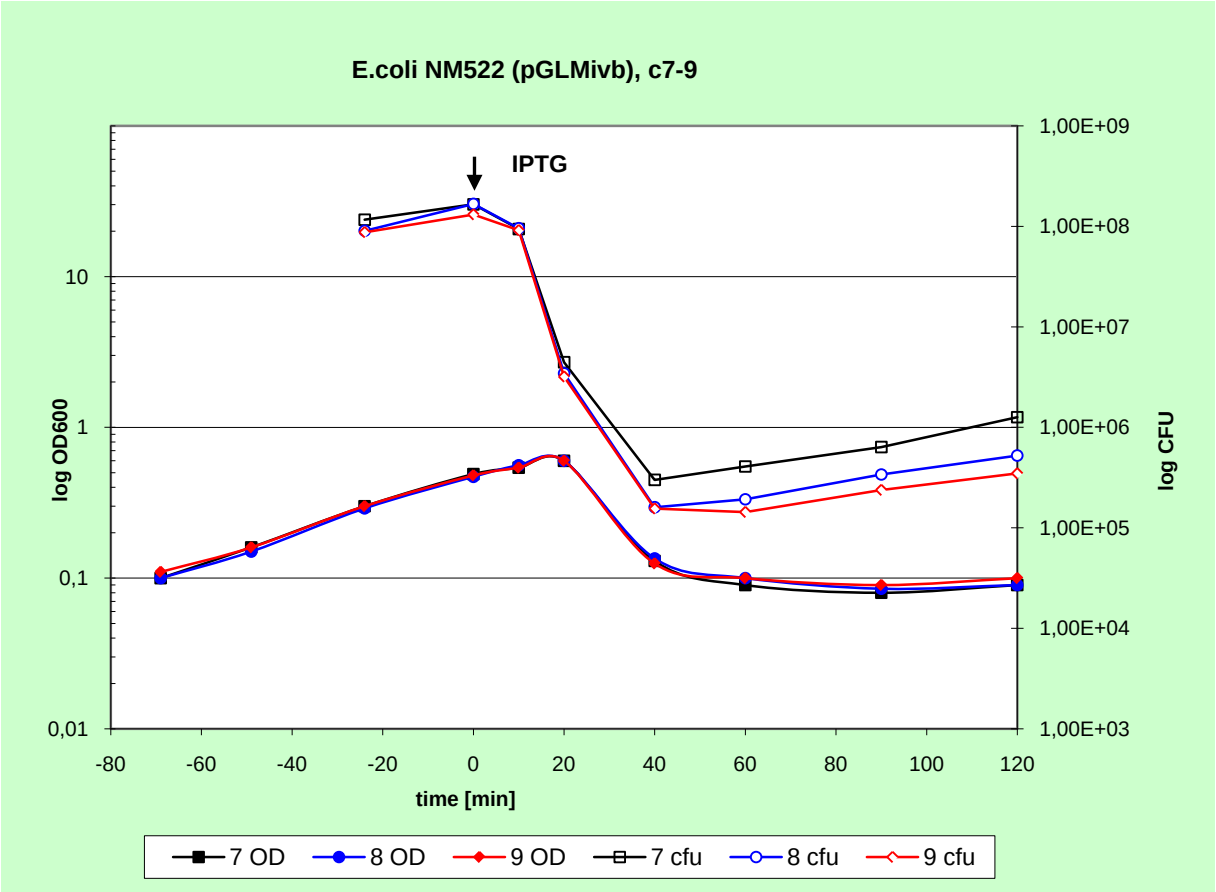

Fig. 39: Growth (OD) and lysis (cfu) curve of E. coli* K12 NM522 *(pGLMivb) clone 7 – 9. *The arrow indicates the time of lysis induction by IPTG addition. Clone 8 shows the best lysis profil.*

cfu	Highest cfu	Lowest cfu	Lysis efficiency
E. coli K12 NM522 (pGLysivb) – positive control	$7{,}87 \times 10^{7}$	$6{,}50 \times 10^{3}$	99,99%
E. coli K12 NM522 (pGLMivb) clone 1	$2{,}66 \times 10^{8}$	$4{,}72 \times 10^{5}$	99,82%
E. coli K12 NM522 (pGLMivb) clone 2	$2{,}61 \times 10^{8}$	$4{,}53 \times 10^{5}$	99,82%
E. coli K12 NM522 (pGLMivb) clone 3	$1{,}93 \times 10^{8}$	$6{,}63 \times 10^{5}$	99,65%
E. coli K12 NM522 (pGLMivb) clone 4	$1{,}23 \times 10^{8}$	$1{,}67 \times 10^{5}$	99,86%
E. coli K12 NM522 (pGLMivb) clone 5	$1{,}87 \times 10^{8}$	$5{,}54 \times 10^{5}$	99,70%
E. coli K12 NM522 (pGLMivb) clone 6	$2{,}40 \times 10^{8}$	$3{,}49 \times 10^{5}$	99,85%
E. coli K12 NM522 (pGLMivb) clone 7	$1{,}66 \times 10^{8}$	$3{,}00 \times 10^{5}$	99,81%
E. coli K12 NM522 (pGLMivb) clone 8	$1{,}67 \times 10^{8}$	$1{,}61 \times 10^{5}$	99,90%
E. coli K12 NM522 (pGLMivb) clone 9	$1{,}31 \times 10^{8}$	$1{,}44 \times 10^{5}$	99,89%

Tab. 5: Lysis efficiency of E. coli* K12 NM522 *(pGLysivb) and E. coli K12 NM522 (pGLMivb)

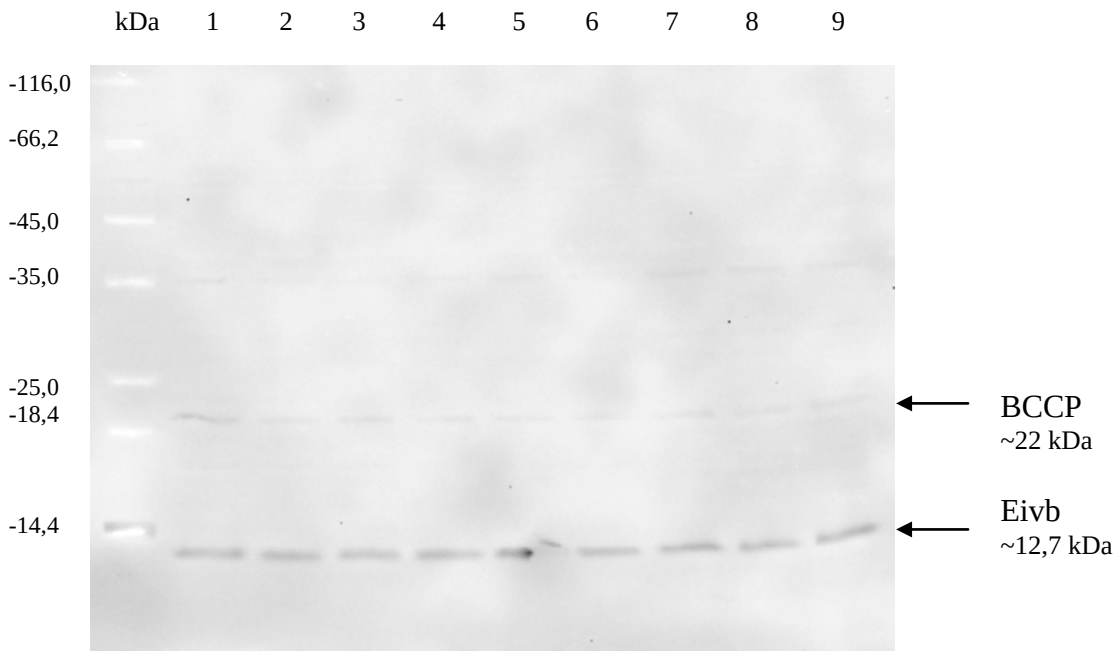

Fig. 40: Western blot showing Eivb protein and cytoplasmic BCCP from lysis consistency study of E. coli* K12 NM522 *(pGLMivb). *1 – 9 = E. coli* K12 NM522 *(pGLMivb) clone 1 – 9, 60 min after lysis induction by IPTG. About the same Eivb expression level is visible in all clones.*

4.2.3 Lysis plasmid – pGULMivb

Plasmid pGULMivb was designed for production of BGs of *Neisseria* species. Plasmid pGULMivb is a modification of pGLysivb where the temperature inducible λpR-cI857 promotor repressor system was exchanged by a chemical inducible LacIq-Repressor-Ptac-Promotor system (lysis-induction with IPTG). Simultaneously the uptake-sequence of *Neisseria* (5'-GCCGTCTGAA-3 ') was introduced by PCR primers. This uptake sequence is necessary to transform a plasmid into *Neisseria* strains.

4.2.3.1 Construction of lysis plasmid pGULMivb

Plasmid pMal-p2x was used as template for amplification of the LacIq-Repressor-Promoter-Ptac-cassette. Restriction sites for XhoI and SbfI and the uptake sequence of *Neisseria* (5’- GCCGTCTGAA- 3’) were introduced by PCR.

Plasmid pGLysivb was digested with XhoI and SbfI to clone the digested PCR fragment using the compatible ends (see Fig. 41).

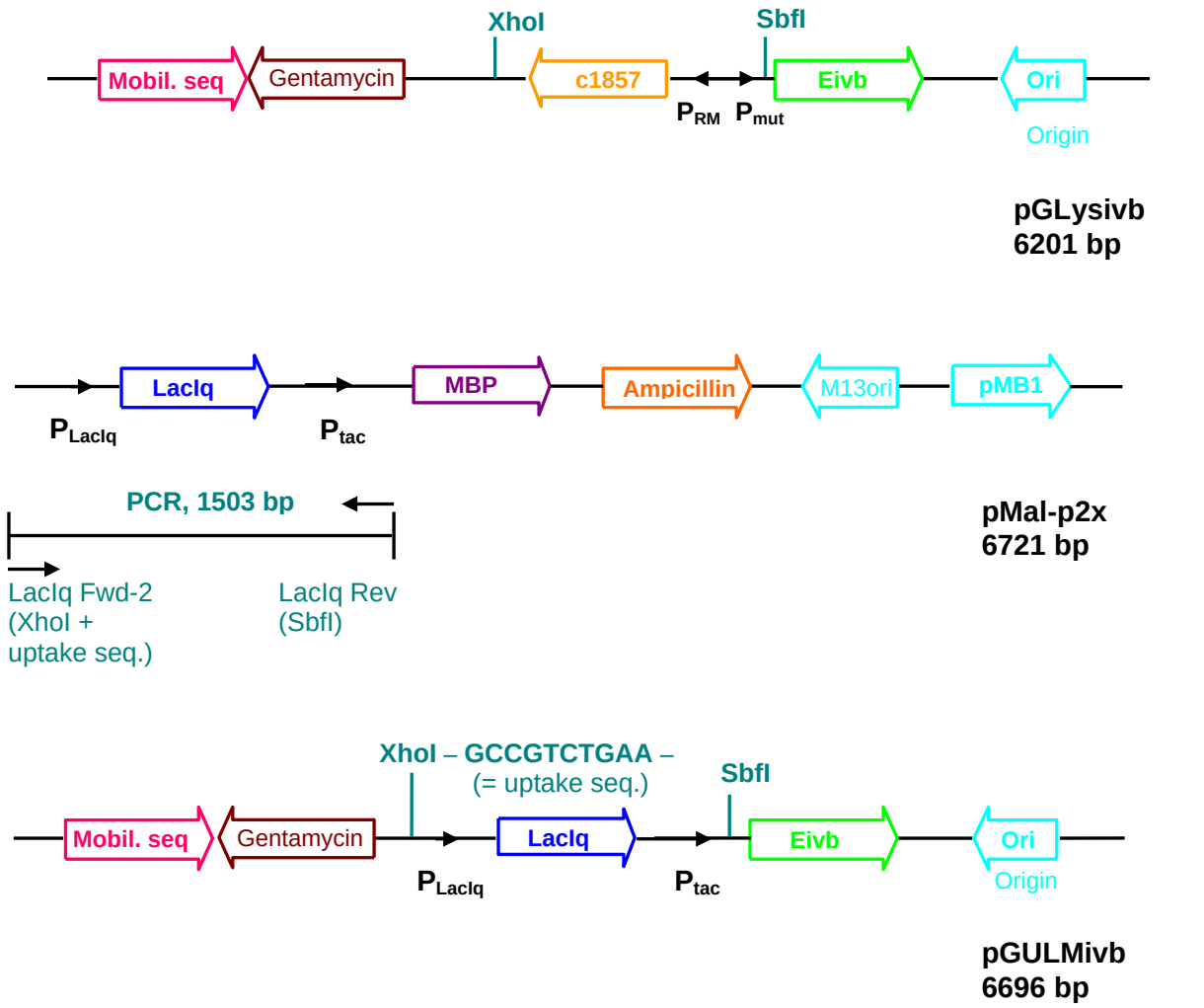

Fig. 41: Cloning strategy of lysis plasmid pGULMivb. *pGLysivb is the backbone plasmid. pMal-p2x is the donor plasmid for the LacIq-repressor-Ptac-promoter cassette. pGULMivb contains the lysis gene Eivb under control of the LacIq-repressor-Ptac-promoter system. Additionally it contains the Neisseria sp."uptake sequence" which was introduced by LacIq Fwd-2 primer.*

PCR was carried out according to standard protocol using:

- Pfu - Polymerase (2,5 u/µl) from Fermentas
- 10x Pfu - buffer (+$MgCl_2$) system

For the PCR following primers were used:

LacIq-XhoI-fwd2:
5` ATA CTC GAG CCG TCT GAA ATT CCG ACA CCA TCG AAT G 3´
Tm = 64°C
LacIq-SbfI-rev: 5` ATA CCT GCA GGA CTG GCT GTT TCC TGT 3´
Tm=56°C

5'GCCGTCTGAA 3'	-10-Base-Pair sequence for Uptake of DNA
….	Restriction sequence
___	Binding region within the insert fragment

After transformation of the ligation mix into *E. coli* K12 C2988J, positive clones were analysed by restriction digests (see Fig. 42) and stored as glycerin stocks.

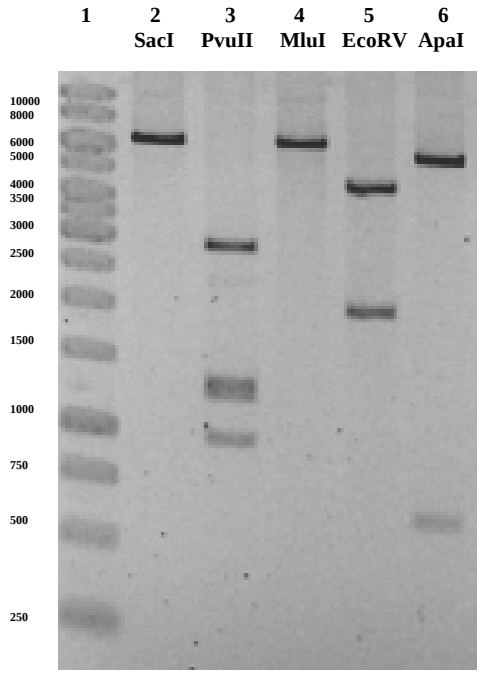

Fig. 42: Control-digest of pGULMivb. *Lane 1: Fermentas GeneRuler 1kb DNA ladder; lane 2: SacI 6696bp correct; lane 3: PvuII 2968/1352/1281/1002/93bp (small fragment not visible) correct; lane 4: MluI 6696bp correct; lane 5: EcoRV 4620/2076bp correct; lane 6: ApaI 6032/664bp correct.*

4.2.3.2 Lysis consistency study of *E. coli* K12 NM522 (pGULMivb)

Bacterial strain *E. coli* K12 NM522 was used for the BG production.Therefore the lysis behavior of pGULMivb in *E. coli* K12 NM522 was studied in a consistency study. 6 different clones of *E. coli* K12 NM522 (pGULMivb) were compared. The clone with the best lysis profil was selected for BG production by fermentation. Experiments are carried out in the nose flasks with 25 ml of LBv and the corresponding antibiotics as described in material and methods.

E. coli K12 NM522 (pGULMivb) clone 6 showed the best lysis profil as shown in Fig. 43 and Tab. 6. Immediately after IPTG addition a decrease in cfu occurred. Lysis end was reached already 40 min after IPTG addition. Further incubation resulted in regeneration of not lysed cells.

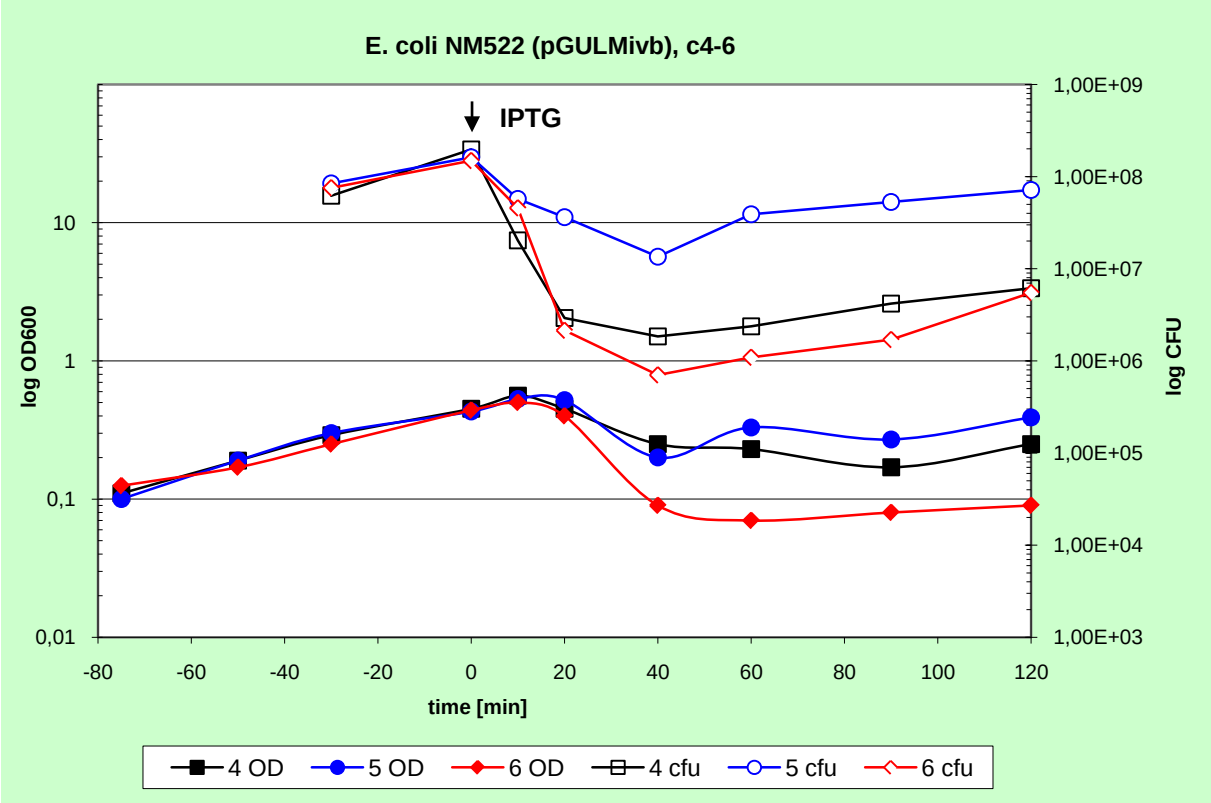

Fig. 43: Growth (OD) and lysis (cfu) curve of E. coli* K12 NM522 *(pGULMivb) clone 4 – 6. *The arrow indicates the time of lysis induction by IPTG addition. Clone 6 shows the best lysis profil.*

cfu	Highest cfu	Lowest cfu	Lysis efficiency
E. coli K12 NM522 (pGLysivb) – positive control	7,87 x 10^7	6,50 x 10^3	99,99%
E.coli K12 NM522 (pGULMivb) clone1	6,66 x 10^7	8,19 x 10^5	98,77%
E.coli K12 NM522 (pGULMivb) clone2	7,84 x 10^7	1,09 x 10^6	98,61%
E.coli K12 NM522 (pGULMivb) clone3	1,41 x 10^8	7,73 x 10^5	99,45%
E.coli K12 NM522 (pGULMivb) clone4	1,97 x 10^8	1,84 x 10^6	99,07%
E.coli K12 NM522 (pGULMivb) clone5	1,62 x 10^8	1,35 x 10^7	91,67%
E.coli K12 NM522 (pGULMivb) clone6	1,49 x 10^8	7,02 x 10^5	99,53%

Tab. 6: Lysis efficiency of E. coli* K12 NM522 *(pGLysivb) and E. coli K12 NM522 (pGULMivb)

A western blot was performed for qualitative analysis of Eivb protein and cytoplasmic BCCP. As shown in Fig. 44, all 6 clones of *E. coli* K12 NM522 (pGULMivb) showed the expression of Eivb protein. Concentration of cytoplasmic BCCP is weakly as it is expelled during the lysis process.

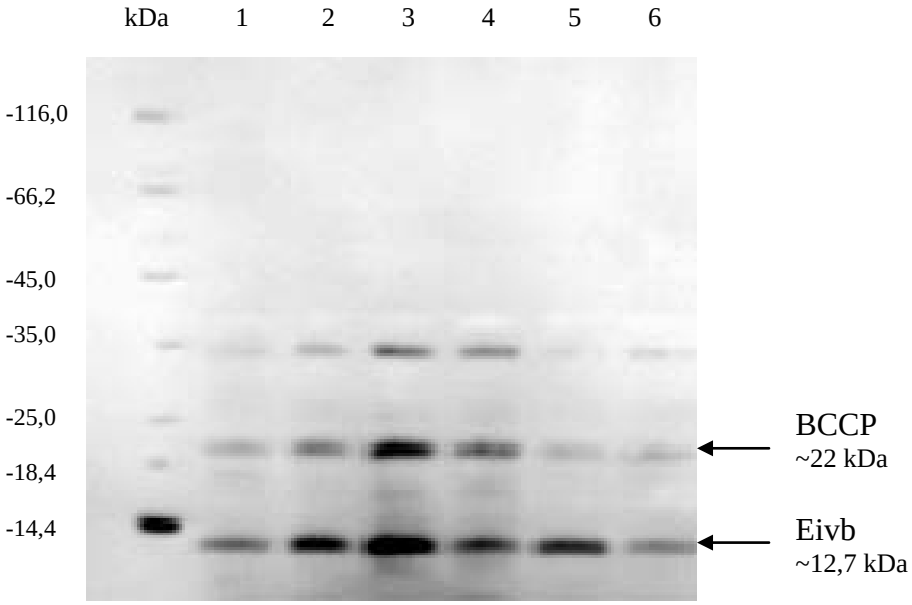

Fig. 44: Western blot showing Eivb protein and cytoplasmic BCCP from lysis consistency study of E. coli* K12 NM522 *(pGULMivb). *1 – 6 = E. coli* K12 NM522 *(pGULMivb) clone 1 – 6, 60 min after lysis induction by IPTG. Eivb expression is visible in all clones.*

Transformation into *Neisseria sp.* was not analysed yet.

5 Discussion

5.1 Bacterial ghosts as carrier of hCG-ß-LTB

The aim of this study was the production of an immunocontraceptive vaccine on the basis of the bacterial ghost system. Former studies of hCG vaccination in women passed the Phase II of clinical trials but generates protective threshold titers in only 60-80% of women. A birth control vaccine has to be effective in more than 90-95% of recipients [2]. Bacterial ghosts function as carrier of foreign antigens and/or as adjuvants. Therefore the bacterial ghost system provides a good possibility to increase the effectiveness.

The first part of this study was the construction of an expression plasmid, pBGK-CGL, which contains the target sequence of hCG-ß-LTB (developed by Dr.Talvar). The new plasmid, pBGK-CGL, is based on pBGKA [14] (see Fig. 43).

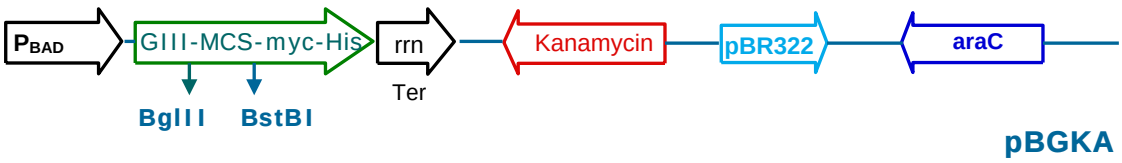

Fig. 43: Linear map of pBGKA.

Therefore pBGK-CGL shows the main properties of the pBAD/GIII system:

- tightly regulated expression of target protein through the L-arabinose induced promoter
- GIII transport system of target protein to the periplasmic space
- Myc-His construct for the detection via anti-Myc and anti-His antibodies

The cloned target gene hCG-ß-LTB was inserted downstream of the GIII gene resulting in a GIII-hCG-ß-LTB-Myc-His fusion protein. GIII as a signal sequence for the targeted transport in the periplasmic space is cleaved by its passage through the inner membrane.

The function of the new plasmid pBGK-CGL, which was transformed in *E. coli* K12 C2988J, was carried out by expression study. The expression of the hCG-ß-LTB-Myc-His fusion protein was detected by western blot analysis (see Fig. 19).

E. coli K12 NM522 was cotransformed with expression plasmid pBGK-CGL and lysis plasmid pGLysivb. The expression and lysis study of *E. coli* K12 NM522 (pBGK-CGL, pGLysivb) showed that hCG-ß-LTB did not influence the bacterial growth (see Fig. 20).
The E-mediated lysis is induced by a shift of temperature from 35-42°C.Fig. 20 showed good lysis efficiency. The expression of the hCG-ß-LTB-myc-His fusion protein was detected by western blot analysis (see Fig. 21). Results of western blot analysis also showed 20 minutes after lysis induction there was an unexpected decrease of protein concentration hCG-ß-LTB. There could be several reasons for that:

- Bacterial strain
- Wrong fermentation conditions (mechanical forces, impaired lysis tunnel formation)
- Impaired GIII transport

Possible solutions:

- New bacterial strain (*E. coli K12 W3110* which is protease deficient)
- New fermentation conditions (large-scale fermentation)
- New transporter system (new cloning strategy)

Therefore *E. coli* K12 W3110 was cotransformed with expression plasmid pBGK-CGL and lysis plasmid pGLysivb. The expression and lysis study of *E. coli* K12 W3110 (pBGK-CGL, pGLysivb) showed that hCG-ß-LTB did not influence the bacterial growth (see Fig. 22).
The E-mediated lysis is induced by a shift of temperature from 35-42°C. Fig. 22 showed good lysis efficiency. The expression of the hCG-ß-LTB-myc-His fusion protein was detected by western blot analysis (see Fig. 23), which showed no protein loss after lysis induction.

The best clones of the tested bacterial strains *E. coli* K12 W3110 (pBGK-CGL, pGLysivb) and *E. coli* K12 NM522 (pBGK-CGL, pGLysivb) were used for the large-scale fermentation in the 30 liter fermeter. Under these conditions also *E. coli* K12 NM522 showed no protein loss after lysis induction (see Fig. 27).

Fermentation of *E. coli* K12 NM522 (pBGK-CGL, pGLysivb) clone 1	
Lysis efficiency	99,998 %
Yield	7890 mg
Particles - / mg	$1{,}05 \times 10^9$
ng hCGß-LTB / µg BG	44,51 ng / µg BG

Fermentation of *E. coli* K12 W3110 (pBGK-CGL, pGLysivb) clone 2	
Lysis efficiency	99,995 %
Yield	9453 mg
Particles - / mg	$1{,}19 \times 10^9$
ng hCGß-LTB / µg BG	49, 75 ng / µg BG

Tab. 6: Results of large scale fermentations *– lysis efficiency, yield, particles / mg, average of ng hCG-ß-LTB / µg B*G.

As shown in Tab. 6 both large scale fermentations indicate similar results. Fermentation of *E. coli* K12 W3110 exhibits greater yield and higher concentrations of hCG-ß-LTB target protein. Lyophilized bacterial ghosts of both large-scale fermentations have been sent to Dr. Talwar (Talwar Research Foundation, New Delhi, India) for further immunological approaches.
Following immunological studies should show if those hCG-ß-BG-vaccine, produced in this work, due to a better efficiency compared to the previous hCG-ß-vaccines.

5.2 Lysis plasmids

Lysis plasmids are essential for the production of bacterial ghosts. The efficiency of lysis mainly depends on the promoter that controls expression of lysis gene E but can also be influenced by other factors like plasmid size, other genes (eg. resistance

genes) and the bacterial system in which you will induce E-mediated lysis. Because of that all lysis plasmids have to be tested after construction.

In this work three lysis plasmids have been constructed:

- pGLysivb-2x
- pGLMivb
- pGULMivb

All three plasmids are derived from pGLysivb.

In pGLysivb-2x the expression of the two lysis genes E is controlled by a temperature inducible λpR-cI857 promoter-repressor system. The idea behind the construction of this plasmid was that two instead of one lysis cassettes should due to higher lysis efficiency as well to a more rapid lysis induction. Unfortunately this was not the case, in contrast the new lysis plasmid showed poor lysis and lysis efficiency compared to pGLysivb (see Fig. 35, Tab. 3). One explanation for these results could be the mutual inhibition of the two E-lysis cassettes.

In case of pGLMivb instead of temperature inducible promoter-repressor system, a Lac-Iq-repressor-Ptac-promoter system was used. Some bacteria show optimal growth by temperatures above 36°C. So a temperature inducible system, which is sensitive to temperature up to 36°C, would not be useful. The pGLMivb showed good lysis efficiency and a fast lysis, but also a rapid regeneration of non-lysed bacteria after the end-point of lysis (see Fig. 39, 40; Tab. 5).

The construction of pGULMivb was based on nearly the same principle as that of pGLMivb. The expression of lysis gene E was controlled by the Lac-Iq-repressor-Ptac-promoter system, since it is known that the optimal growth of *Neisseria sp*. is at higher temperatures. Additionally an uptake sequence was introduced via primers (see Fig. 41) to enable the admission of the pGULMivb during transformation.
The cloning strategy, cloning and lysis analysis was performed in this work. The pGULMivb showed nearly the same results like pGLMivb. The pGULMivb showed

also good lysis efficiency, fast lysis and a rapid regeneration of non-lysed bacteria after the end-point of lysis (see Fig. 43, 44; Tab. 6).

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7 Appendix

Overview

Backbone plasmids:

Plasmid	Expression cassettes	Resistance	Origin	Size [Kb]	References
pBGKA	P_{BAD}-gIII-MCS-myc-His	Kanamycin	pBR322	4,04	[14]
pDrive-hCG-ß-LTB	P_{lac}-hCG-ß-LTB	Kanamycin / Ampicillin	pMB1	4,53	[51]
pMal-p2x	LacIq-P_{tac}-malE	Ampicillin	pMB1	6,721	New England Biolabs

Expression plasmids:

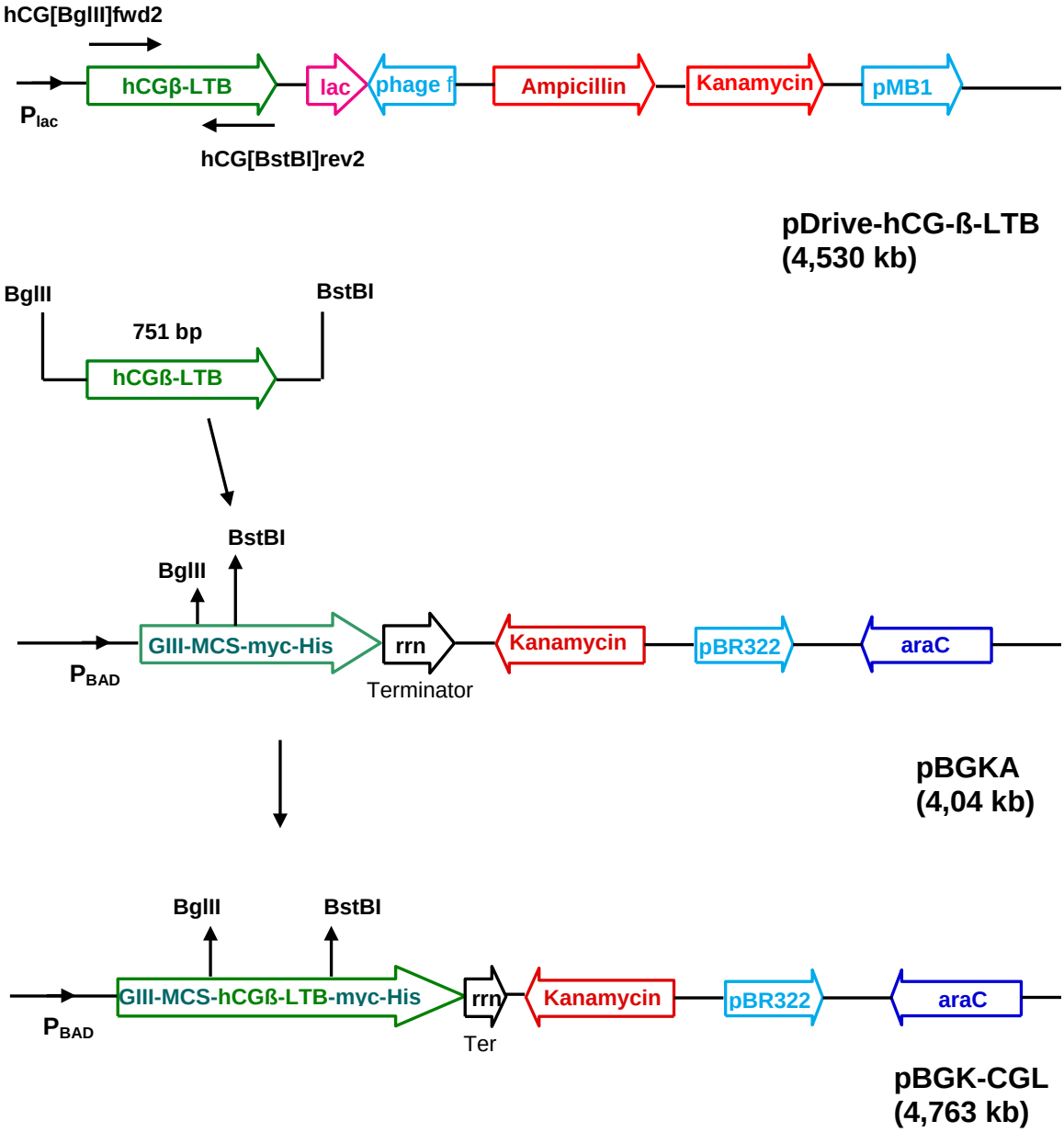
Plasmid	Expression cassettes	Resistance	Origin	Size [Kb]	References
pBGK-CGL	P_{BAD}-gIII-MCS-hCG-ß-LTB-myc-His	Kanamycin	pBR322	4,763	this work

Lysis plasmids:

Plasmid	Expression cassettes	Resistance	Origin	Size [Kb]	References
pGLysivb	cI857- λP_{Rmut} -Eivb	Gentamycin	Rep	6,201	[32]
pGLysivb-2x (B)	cI857- λP_{Rmut} -Eivb / cI857- λP_{Rmut} -Eivb	Gentamycin	Rep	7,168	this work

pGLysivb-2x (A)	cI857- λP_{Rmut} -Eivb / cI857- λP_{Rmut} -Eivb	Gentamycin	Rep	7,274	this work (not working)
pGLMivb	LacIq-P_{tac}-Eivb	Gentamycin	Rep	6,68	this work
pGULMivb	Uptake seq. / LacIq-P_{tac}-Eivb	Gentamycin	Rep	6,696	this work
pGELys	cI857- λP_{Rmut} -Eivb	Gentamycin	Rep	5,06	[10]
pGES	cI857- λP_{Rmut} -Eivb	Gentamycin	Rep	4,482	this work (not working)
pGLNIc	cI857- λP_{Rmut} -Eivb / LacIq-P_{lac}-Snuc	Gentamycin	Rep	8,309	[52]
pGLNIs	cI857- λP_{Rmut} -Eivb / LacIq-P_{lac}-Snuc	Gentamycin	Rep	7,168	this work (not working)

Construction of plasmid pBGK-CGL



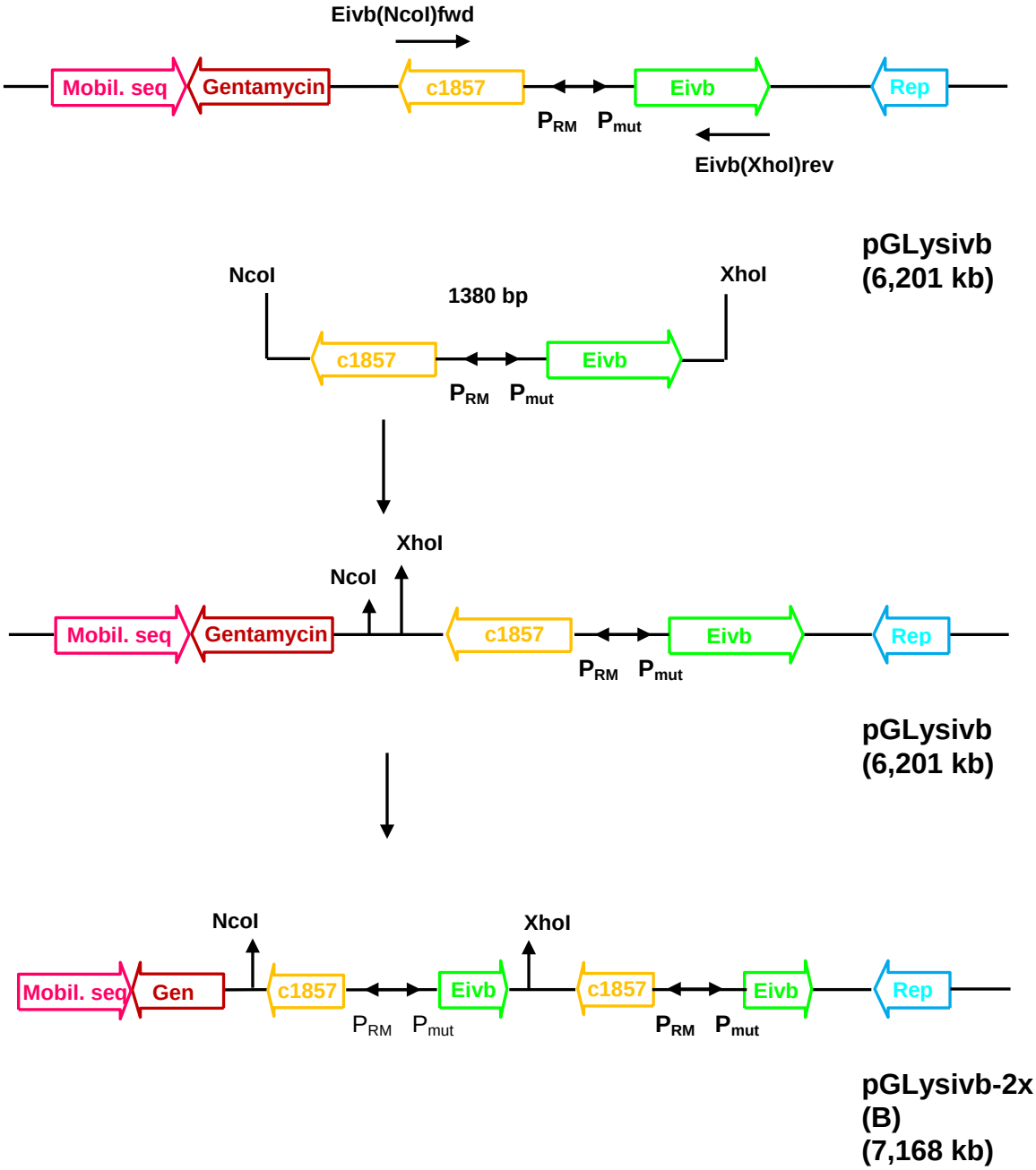
PCR primer:

hCG[BglII]fwd2: 5` AATAG ATC TCC AAG GAC CCG CTT CGG 3´

hCG[BstBI]rev2: 5` AATT TCG AAA GTT TTC CAT ACT GAT TGC CGC A 3´

…. Restriction sequence
___ Binding region within the insert fragment

Construction of plasmid pGLysivb-2x (B - strategy)



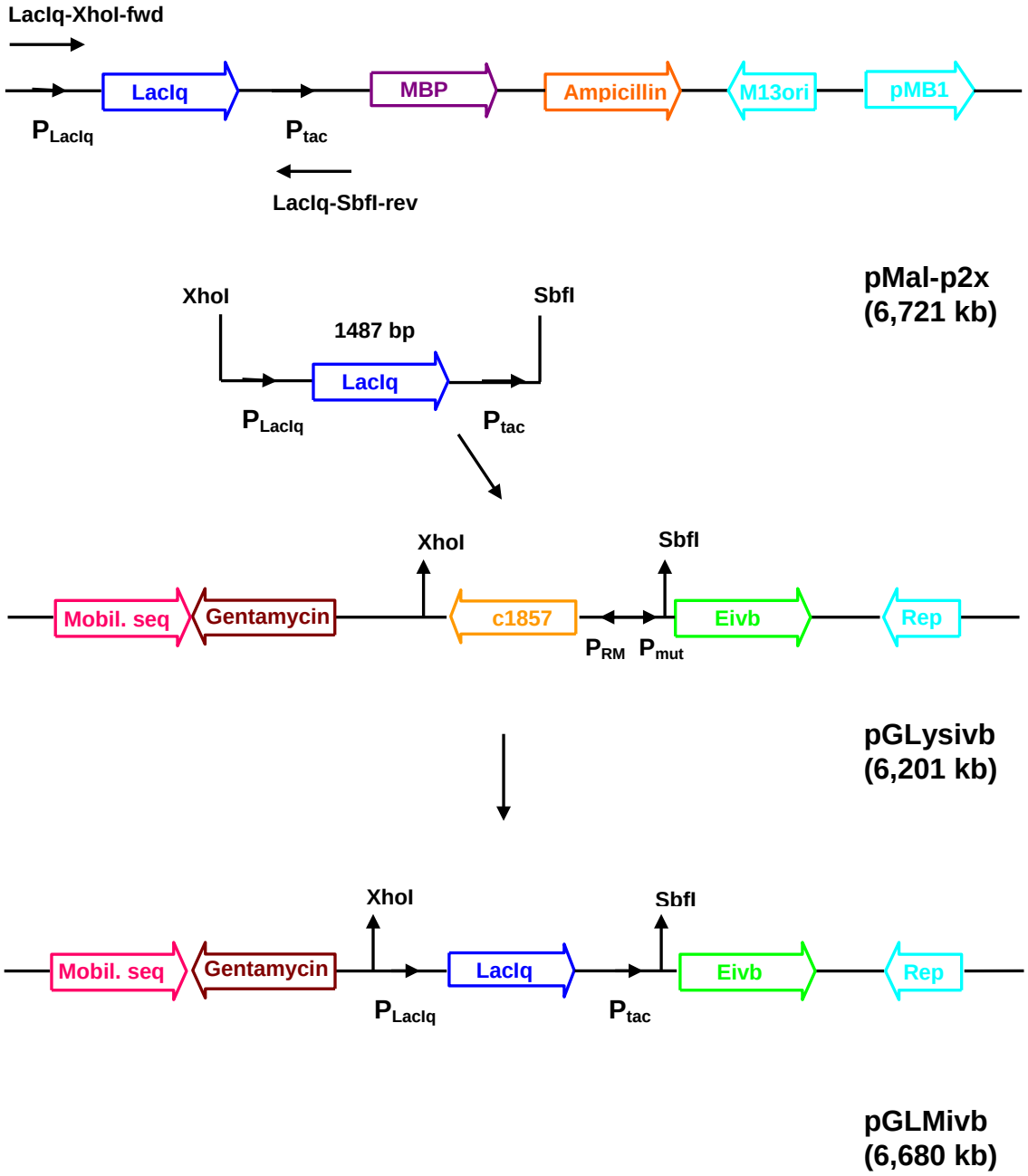
PCR primer:

Eivb(NcoI)fwd: 5` AAT CCA TGG TCA GCC AAA CGT CTC TTC 3´

Eivb(XhoI)rev: 5` AAT CTC GAG TCA TTC GTG CCA TTC GAT T 3´

…. Restriction sequence
___ Binding region within the insert fragment

Construction of plasmid pGLMivb



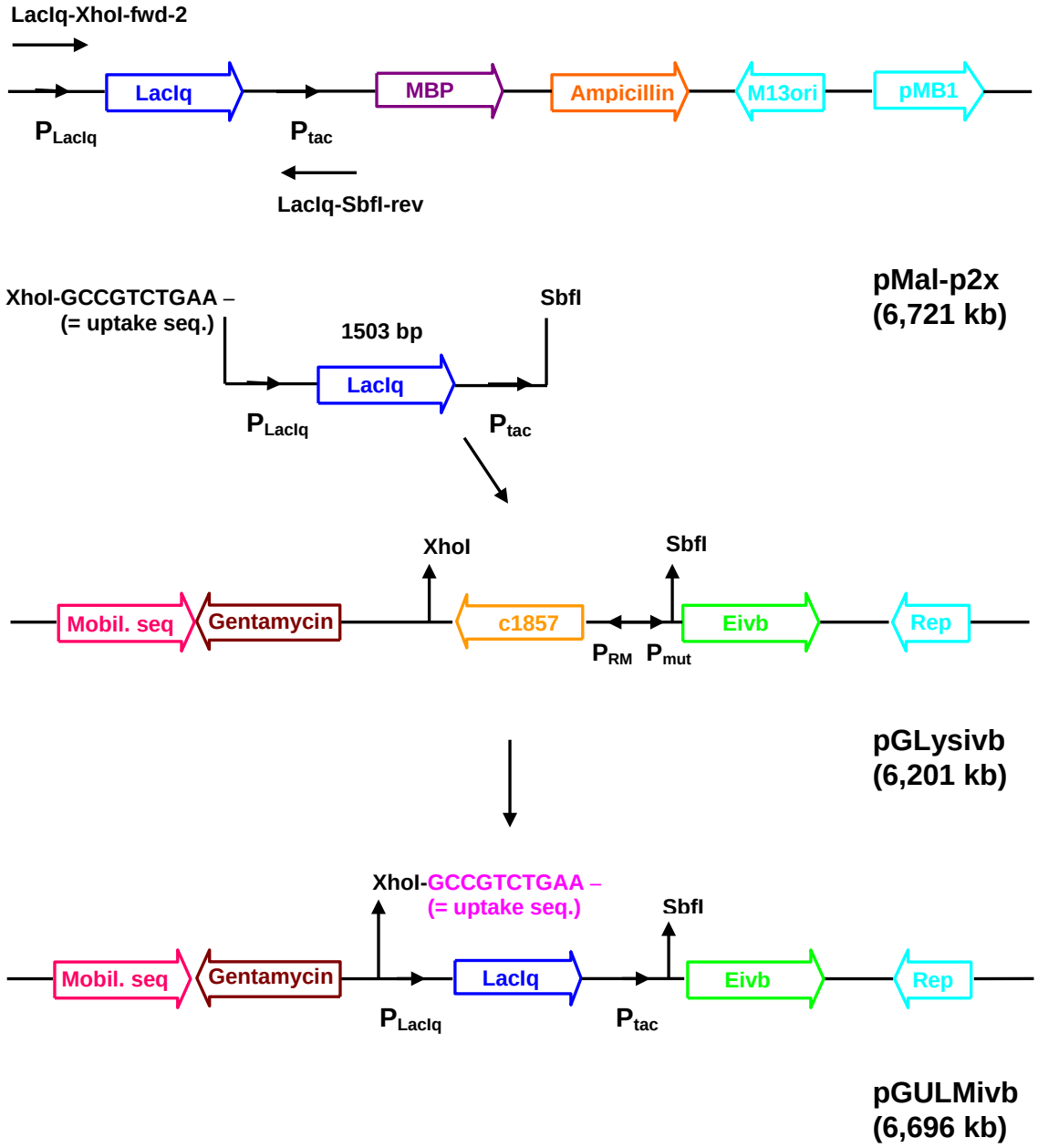
PCR primer:

LacIq-XhoI-fwd: 5` ATA CTC GAG CAC CAT CGA ATG GTG CAA A 3´

LacIq-SbfI-rev: 5` ATA CCT GCA GGA CTG GCT GTT TCC TGT 3´

…. Restriction sequence
___ Binding region within the insert fragment

Construction of plasmid pGULMivb



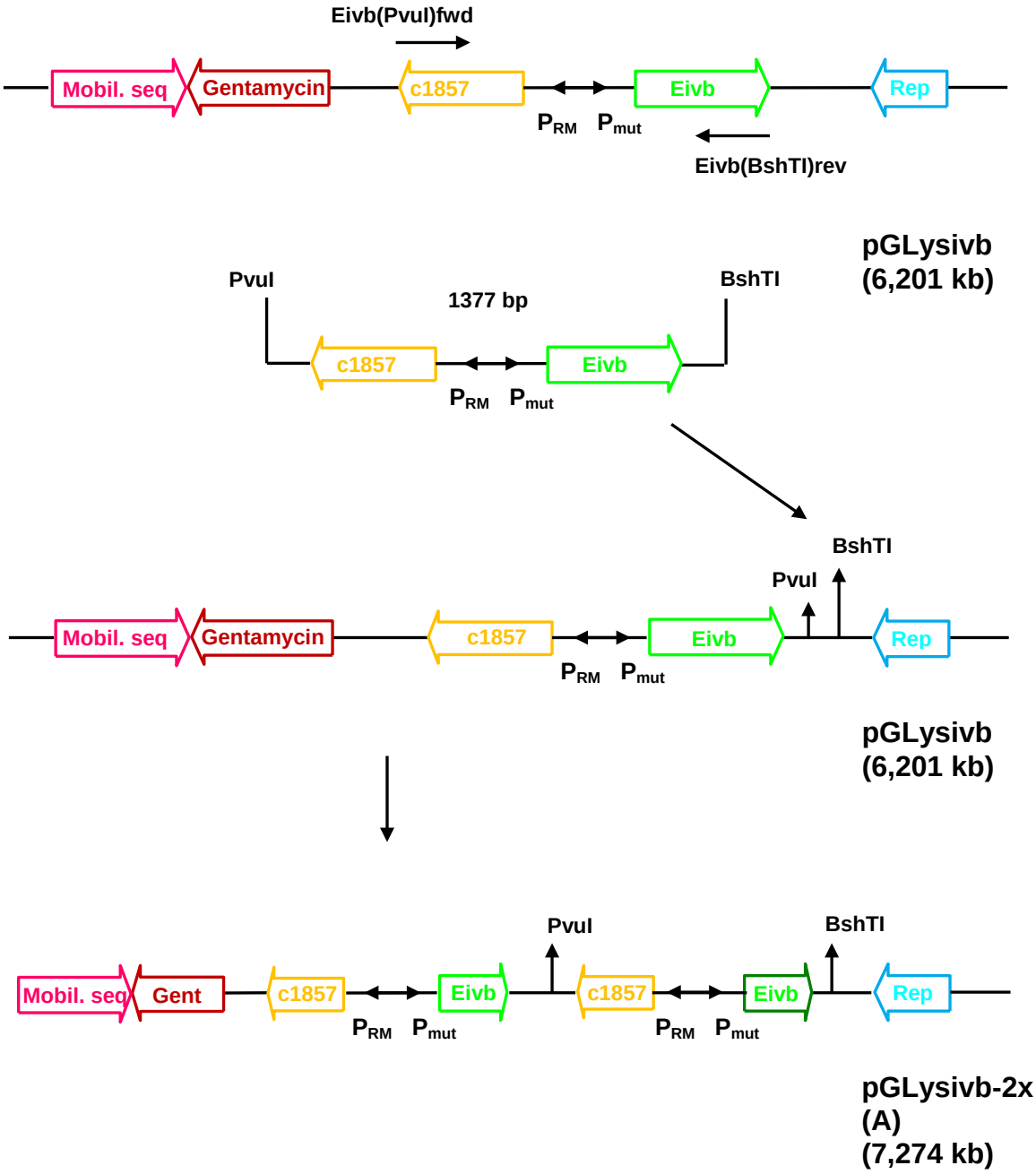
PCR primer:

LacIq-XhoI-fwd-2:
5` ATA CTC GAG CCG TCT GAA ATT CCG ACA CCA TCG AAT G 3´

LacIq-SbfI-rev:
5` ATA CCT GCA GGA CTG GCT GTT TCC TGT 3´

5'GCCGTCTGAA 3' -10-Base-Pair sequence for Uptake of DNA
.... Restriction sequence
___ Binding region within the insert fragment

Construction of plasmid pGLysivb-2x (A – strategy, not working)



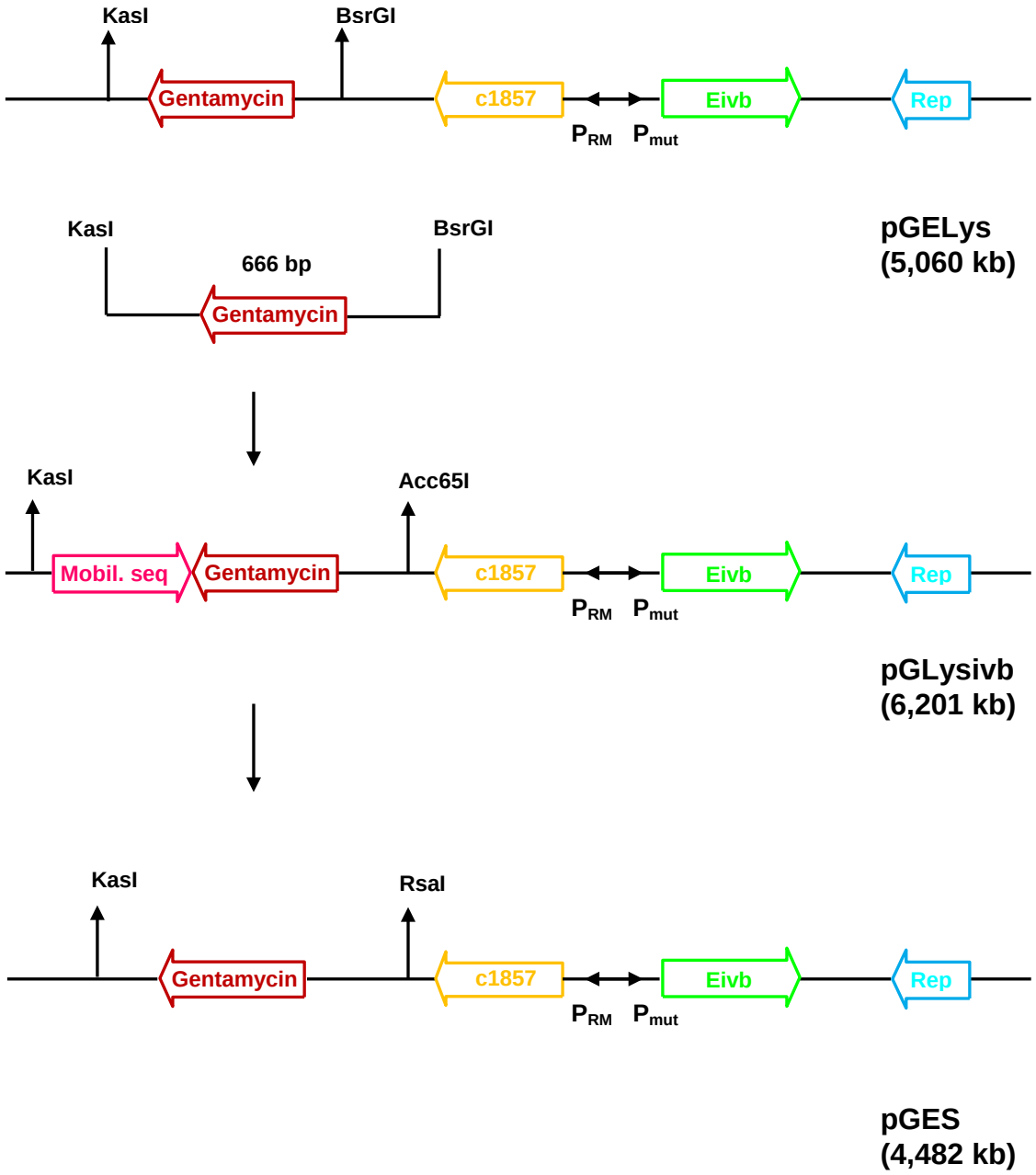
PCR primer:

Eivb(PvuI)fwd: 5` AATT CGA TCG TCA GCC AAA CGT CTC TTC 3´

Eivb(AgeI)rev: 5` AAT ACC GGT TCA TTC GTG CCA TTC GAT T 3´
AgeI = BshTI

…. Restriction sequence
___ Binding region within the insert fragment

Construction of plasmid pGES (not working)

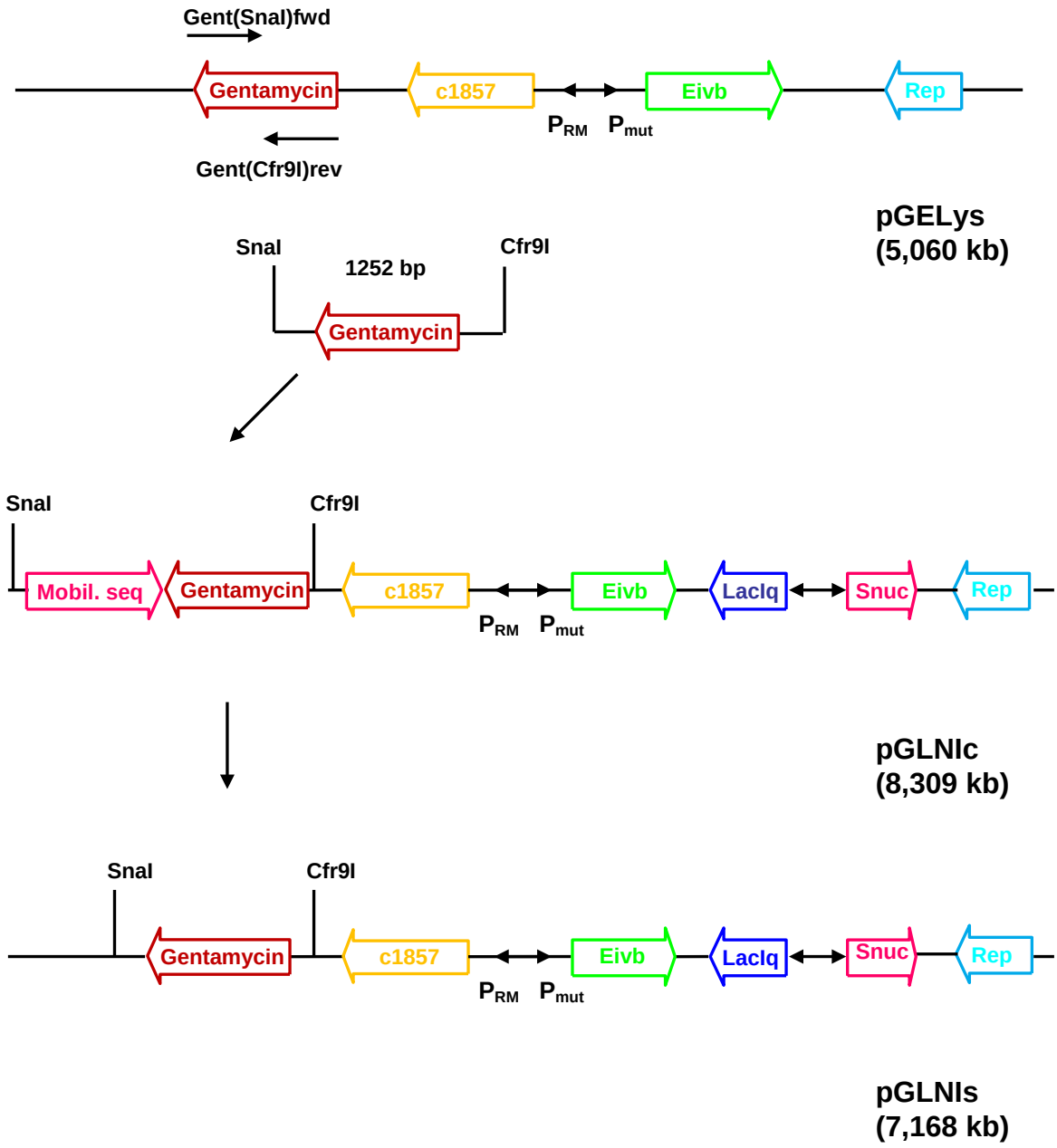


Plasmid pGELys is used as insert for cloning

Insert pGELys [5060bp] ⤫ KasI / BsrGI

4394bp / **666bp**

Construction of plasmid pGLNIs (not working)



PCR primer:

Gent (SnaI)Fwd: 5` ATA GTA TAC TTA GGT GGC GGT ACT TGG GTC3´
[SnaI=BstZ17I]

Gent (Cfr9I)Rev: 5` ATA CCC GGG CTG CAG GAA TTC GA 3´
[Cfr9I=SmaI]

…. Restriction sequence
___ Binding region within the insert fragment

8 Curriculum vitae

Personal data:

Name	Ivana Hodul
Born	08.05.1979, Myjava, Slovakia
Citizenship	Slovakia

Education:

1985 – 1993	Primary / Secondary school, Krajne, Slovakia
1993 – 1997	High school – Gymnasium Myjava, Slovakia
1997 – 1998	Study of Biology, Komensky University Bratislava, Slovakia
1998 – 2000	Study of Biology, K. F. University Graz
2000 – 2003	Study of Medicin, K. F. University Graz
2003 – 2008	Study of Biology, University Vienna
2008 – 2009	Master's thesis at the Department of medicinal / pharmaceutical chemistry, University Vienna